UNIVERSIDADE FEDERAL DO PARANÁ

IVO ALBERTO BORGHETTI

DESENVOLVIMENTO DE UM CONTROLE EXTERNO POSITIVO DO TIPO *VIRAL- LIKE - PARTICLE* PARA UTILIZAÇÃO EM UM TESTE DIAGNÓSTICO DE ARBOVIROSES EMERGENTES

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Tese apresentada ao curso Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Engenharia de Bioprocessos e Biotecnilogia.

Orientador: Prof. Dr. Marco Aurélio Krieger

Coorientadora: Drª. Rita de Cássia Pontello Rampazzo

Coorientadora: Profª. Drª. Vanete Thomaz Soccol

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **IVO ALBERTO BORGHETTI** intitulada: **Desenvolvimento de um controle externo positivo do tipo viral - LIKE - Particle para utilização em um teste diagnóstico de arboviroses emergentes**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 22 de Junho de 2018.

MARCO AURELIO KRIEGER
Presidente da Banca Examinadora

DEUSILENE SOUZA VIEIRA DALLACQUA
Avaliador Externo

VANETE THOMAZ SOCCOL
Avaliador Interno

LUCIANA PORTO DE SOUZA VANDENBERGHE
Avaliador Interno

RITA DE CASSIA PONTELLO RAMPAZZO
Avaliador Externo

Dedico este trabalho primeiramente a Deus, por ser essencial em minha vida, autor de meu destino, meu guia, socorro presente na hora da angústia, ao meu pai Severino Ivo Borghetti (*in memorian*), minha mãe Ires Anna Borghetti e aos meus irmãos que sempre apostaram e me apoiaram nesta nova etapa de minha vida

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"Para nós os grandes homens não são aqueles que resolveram os problemas, mas aqueles que os descobriram."

(Albert Scweitzer)

RESUMO

Os arbovírus como dengue, Zika e chikungunya têm se proliferado principalmente em regiões tropicais e subtropicais por conta de condições climáticas, ambientais e falta de saneamento. Os sinais e sintomas das arboviroses citadas podem ser bastante semelhantes e representam um problema para o diagnóstico clínico. Assim, o diagnóstico laboratorial é imprescindível para definição das condutas terapêuticas a serem adotadas no tratamento dos pacientes suspeitos. Os testes mais usados no diagnóstico laboratorial são os sorológicos e moleculares, sendo que os testes moleculares apresentam a vantagem de serem mais sensíveis e específicos, enquanto que os sorológicos têm a desvantagem de apresentar riscos de reações cruzadas, uma vez que esses vírus tem genomas bastante semelhantes. O objetivo deste trabalho foi construir um controle externo positivo único contendo sequencias dos arbovírus dengue, Zika e chikungunya e de um transcrito humano específico para validar as reações do kit BIOMOL ZDC. A construção do controle foi feita utilizando técnicas de engenharia genética, foi desenhada uma sequência sintética única contendo fragmentos de todos os alvos do kit, e esta foi sintetizada e clonada em um sistema de expressão conhecido, contendo o promotor T7 e o fago MS2 modificado. Após a expressão e purificação foi feita a extração do RNA das partículas e a amplificação na RT- qPCR. Na análise verificou-se que todos os alvos do kit kit BIOMOL ZDC presentes no controle construído foram amplificados, validando todo o processo desde a extração, a transcrição reversa até a reação de PCR. Portanto, o controle construído atendeu aos objetivos deste projeto e desta forma o kit BIOMOL ZDC que já estava sendo usado para pesquisa no Brasil sem a presença do controle positivo, passou a ser utilizado com o módulo completo pelos mesmos laboratórios que obtiveram resultados semelhantes. Portanto, este produto (kit com o controle), desenvolvido com tecnologia nacional é biosseguro, tendo custo menor e alto valor agregado, e potencial para ser utilizado a fim de atender as necessidades da saúde pública brasileira.

Palavras-chave**:** arbovírus, controle externo, diagnóstico molecular, RT-qPCR.

ABSTRACT

The arboviruses such as dengue, Zika and chikungunya have proliferated mainly in tropical and subtropical regions due to climatic and environmental conditions and lack of sanitation. The signs and symptoms of the aforementioned arboviruses can be very similar and represent a problem for the clinical diagnosis. Thus, the laboratory diagnosis is essential for defining the therapeutic behaviors to be adopted in the treatment of suspected patients. The most commonly used tests in laboratory diagnosis are serological and molecular tests; molecular tests have the advantage of being more sensitive and specific, whereas serological tests have the disadvantage of presenting cross-reactivity risks, since these viruses have very similar genomes. The objective of this work was to construct a single positive external control containing dengue, Zika and chikungunya arbovirus sequences and a specific human transcript to validate the reactions of the BIOMOL ZDC kit. Construction of the control was done using genetic engineering techniques, a single synthetic sequence containing fragments of all kit targets was drawn and synthesized and cloned into a known expression system containing the T7 promoter and the modified MS2 phage. After the expression and purification, the RNA extraction from the particles and the amplification in the RT- qPCR were made. In the analysis it was verified that all the targets of the BIOMOL ZDC kit present in the constructed control were amplified validating the whole process from the extraction, the reverse transcription until the PCR reaction. Therefore, the constructed control met the objectives of this project and in this way the BIOMOL ZDC kit that was already being used for research in Brazil without the presence of the positive control was used with the complete module by the same laboratories that obtained similar results. Therefore, this product (kit with the control), developed with national technology is biosecure, having a lower cost and high added value, having potential to be used to meet the needs of Brazilian public health.

Keywords: arbovirus, external control, molecular diagnosis, RT- qPCR.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

cDNA	- DNA complementar
CI	- Controle Interno
CP	- Controle Positivo
Ct	- "*Cycle threshold*" -
SD	- Desvio-padrão
DENV1	- dengue vírus – sorotipo 1
DENV2	- dengue vírus – sorotipo 2
DENV3	- dengue vírus – sorotipo 3
DENV4	- dengue vírus – sorotipo 4
DF	- dengue "*Fever"*
DHF	- dengue *Hemorragic Fever*
DNA	- Ácido desoxirribonucleico
DNase	- desoxirribonuclease
DNTP(s)	- desoxirribonunucleosídeo triposfato
EDTA	- ácido etileno-diamino-tetracético-
HCV	- Vírus da Hepatite C
HIV	- Vírus da Imunodeficiência Humana
HBV	- Vírus da hepatite B
PCR	- Reação em cadeia pela polimerase
RNA	- ácido ribonucleico
RNase	- ribonuclease
RT- PCR	- *Reverse Transcription-polymerase Chain Reaction*
MS2	- Bacteriófago MS2
Aids	- Síndrome da imunodeficiência adquirida
mL	- Mililitro
μL	- Microlitro
μm	- Micrômetro
Mm	- Milímetro
P.A.	- Para análise
RT	- *Transcriptase reverse*

ANVISA	- Agência Nacional de Vigilância Sanitária
D.O	- densidade optica
Nm	- nanômetro
IPTG	- Isopropyl – β-D-1- thiogalactopyranoside
IOC	- Instituto Oswaldo Cruz
PEG	- Polietilenoglicol
mmM	- milimolar
°C	- graus celsius
VLP	- "*Virus Like Particles"*
G	- gramas
μg	- micrograma
RPM	- rotações por minuto
NaCl	- cloreto de sódio
$MgSO_4.7H_2O$	- sulfato de magnésio heptahidratado
pH	- potencial hidrogeniônico
L.B	- Luria Bertani
UFPR	- Universidade Federal do Paranál
IBMP	- Instituto de Biologia Molecular do Paraná
INTEC	- Incubadora Tecnológica do Paraná
FIOCRUZ	- Fundação Oswaldo Cruz
PPM	- Parte por milhão
LACEN	- Laboratório Central de Saúde Pública
KB	- Kilobase
SS	- Sequência Sintética
MS	- Ministério da Saúde
NAT	- Testes de ácidos nucleicos
SUS	- Sistema único de saúde
ZIKV	- Zika vírus
CHIKV	- Chikungunya vírus
TAQ	- "*Thermus Aquaticus Polymerase"*
ESPIN	- Emergência em saúde pública de importância nacional
AP	- Ampá
BA	- Bahia

ELISA	- *Enzyme-Linked Immunosorbent Assay*
GAPDH	- Glyceraldehyde-3-phosphate desidrogenase
UBQ	- Ubiquitina
PGK	- Fosfoglicerato kinase
RO	- Rondônia
PAHO	- *Pan American Health Organization*
SGB	- *Syndrome of Guillain-Barré*
WHO	- *World Health Organization*

SUMÁRIO

1 INTRODUÇÃO

O Instituto de Biologia Molecular do Paraná (IBMP) foi criado em 1999 através de uma parceria entre a Fundação Oswaldo Cruz (FIOCRUZ) e o Governo do Estado do Paraná sendo que as suas atividades se iniciaram de maneira oficial em 2001.

Desta forma, o Ministério da Saúde (MS) alocou o IBMP como parte de um grupo responsável pela implantação de testes de diagnóstico molecular como o Testes de Ácidos Nucleicos (NAT) para a hemorrede brasileira, destinado, incialmente, à detecção de material genético dos vírus da imunodeficiência humana (HIV), hepatite C (HCV) e, atualmente, também a hepatite B (HBV).

Hoje em dia, o IBMP fornece o módulo de amplificação do teste de diagnóstico molecular NAT destinado a detecção de três vírus, imunodeficiência humana (HIV), hepatite B (HBV) e hepatite C (HCV) para triar todas as bolsas de sangue que circulam na hemorrede pública Brasileira.

Além disso, em seu portifólio o IBMP investe no Desenvolvimento e Inovação buscando soluções para os problemas enfrentados pelo Brasil especialmente aqueles ligados ao Sistema Único de Saúde (SUS).

Dentre os produtos relacionados ao setor de inovação do IBMP, foi desenvolvido no final de 2015, por conta de uma grave epidemia causada por um vírus pouco conhecido, o Zika vírus (ZIKV), um teste capaz de detectar e diferenciar três arbovírus que possuem sinais e sintomas bastante semelhantes, mas com consequências bastante distintas usando *one step reverse transcriptase polymerase chain reaction* em tempo real (RT-qPCR).

O kit nomeado ZDC Biomol é capaz de promover o diagnóstico de ZIKV, dengue (sorotipos: DENV-1, DENV-2, DENV-3 e DENV-4) e chikungunya (CHIK) através de uma reação que acontece em duas etapas. Na primeira a enzima transcriptase reversa gera DNA complementar (cDNA) e na segunda o cDNA é amplificado na reação de PCR dependente de *Thermus aquaticus polymerase* (TAQ), oligonucleotídeos, cofator, sondas e nuleotídeos.

Após o desenvolvimento do kit de tamanha complexidade, o próprio Instituto e os usuários perceberam uma lacuna que não havia sido preenchida, o kit não o possuía um controle externo positivo, levando a sérias reclamações. Os controles positivos são exógenos e o seu DNA ou RNA é extraído paralelamente às amostras clínicas em teste e na reação de PCR são amplificados em outro poço de reação. Desta forma, “dizer” que a reação estava sendo realizada em condições ótimas desde a extração até a amplificação” continuava sendo uma interrogação pois, não era possível validar as rotinas laboratoriais.

Assim, para solucionar a lacuna apresentada muitas estratégias poderiam ser usadas. Quando se pensa em pesquisa básica poderia-se trabalhar com plasmídeos contendo a sequência de interesse, amplicons, transcritos, cDNA para atuar como controle externo positivo da reação.

Entretanto, os exemplos citados acima não atendem às normas da Agência Nacional de Vigilância Sanitária (ANVISA) e, por isso, uma nova proposta foi fundamentada para a construção de um controle externo positivo.

A Anvisa descarta as soluções da pesquisa básica para o desenvolvimento de controles, por motivos óbvios, como a possibilidade de haver mutações, erros de amplificação, erros na transcrição reversa, erros na transcrição, fácil degradação.

Finalmente, levantou-se a hipótese que não deixaria o produto mais caro e que legalmente seria aceita. Essa solução apresenta as vantagens de fácil produção quando as condições ótimas estão estabelecidas e ainda poderia servir de controle para todas as reações do módulo amplificando todos os alvos, transparecendo um paciente coinfectado com todas as doenças nas reações específicas. Então, o objetivo deste trabalho foi desenvolver uma “*viral-like-particle”* derivada do bacteriófago MS2, contendo sequências específicas de cada um dos alvos em estudo. Essas partículas foram usadas para validar a metodologia estabelecida controlando o processo por inteiro, a extração de RNA, a transcrição reversa e a amplificação dos alvos.

A produção do controle externo positivo foi realizada no Instituto de Biologia Molecular do Paraná e a validação também aconteceu no mesmo.

Entretanto, desafiamos outros laboratórios a realizar testes com o controle positivo e obtivemos resultados semelhantes.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Desenvolver um controle externo positivo do tipo "*viral-like-particle"* (controle positivo) para compor um kit diagnóstico de arboviroses emergentes e reemergentes comprovar por RT- qPCR que o modelo estabelecido é capaz de ser amplificado para os quatro sorotipos do vírus da dengue (DENV-1, DENV-2, DENV-3 e DENV-4); Zika (ZIKV), chikungunya (CHIKV) e para o controle interno humano em reações do tipo multiplex no kit Biomol ZDC.

2.2 OBJETIVOS ESPECÍFICOS

Desenhar uma sequência sintética baseando-se em genomas de ZIKV, DENV-1, DENV-2, DENV-3, DENV-4 e CHIKV e do genoma humano obtidas em banco de dados internacionais.

Solicitar a síntese da sequência de interesse e clonagem da mesma no vetor de expressão pET47b(+)-MS2.

Confirmar por sequenciamento a presença da sequência sintética clonada no plasmídeo vetor pET47b(+)-MS2.

Avaliar o controle positivo MS2-ZDC desde a extração de RNA até a amplificação em RT-qPCR no Kit Biomol ZDC.

Avaliar a estabilidade do controle positivo MS2-ZDC de acordo com o tempo de estocagem em temperatura de -20°C

Avaliar a estabilidade do controle positivo MS2-ZDC em diferentes temperaturas antes do processo de extração de RNA.

Utilizar kit Biomol ZDC completo, ou seja, módulo de amplificação e controle, para testar amostras de pacientes suspeitos de infecção causada por arbovírus na região da Amazônia Ocidental em um estudo clínico laboratorial e comprovar a aplicabilidade do controle positivo.

Avaliar o padrão de amplificação de controles positivo e das amostras testadas provenientes da região da Amazônia Ocidental.
Expressar e purificar o controle positivo MS2-ZDC a partir do plasmídeo vetor pET47b(+)-MS2 com a sequência sintética.

3 REVISÃO DE LITERATURA

3.1 GENERALIDADES

As doenças emergentes estão relacionadas com o surgimento ou a identificação de um agente infeccioso e as doenças reemergentes indicam mudança no comportamento epidemiológico de doenças já conhecidas, que haviam sido controladas, mas que voltaram a representar ameaça a saúde. Estas doenças como a dengue, Zika, febre amarela, "*West Nile"*, hantavirus, leptospirose, leishmaniose e doença de Chagas estão diretamente relacionados com o comportamento humano especialmente, com a globalização, urbanização, alterações climáticas, problemas sociais e econômicos[1,2].

No Brasil, os fatores mais importantes para o avanço de zoonoses relacionam-se ao aumento da fronteira agropecuária, construção de hidroelétricas, regiões densamente povoadas e com infraestrutura sanitária inadequada, favorecendo o ciclo parasita-hospedeiro-vetor[1].

Os vírus RNA patogênicos são potencialmente o grupo mais importante envolvido na transmissão de doenças zoonóticas e representam um desafio para o controle global de doenças e incluem não só as espécies virais desconhecidas como também as que são conhecidas e continuam sendo um problema contínuo para a saúde humana e animal[3]. A diversidade biológica desses agentes e as taxas de adaptação rápida tornam o grupo de difícil eliminação mesmo com desenvolvimento tecnológico farmacêutico e médico[3].

3.2 ARBOVÍRUS

Os arbovírus (vírus transmitidos por artrópodes) são taxonomicamente diversos, mas compartilham um ciclo de transmissão entre hospedeiros vertebrados e vetores artrópodes. Todas as espécies de arbovírus, exceto uma, pertencem a uma das cinco famílias de vírus de RNA, sugerindo altas taxas de mutação que podem inclusive ser um pré-requisito para entrar em um ciclo de replicação alternada nos diferentes ambientes representados por animais vertebrados e invertebrados. Até o momento mais de 500 espécies de arbovírus foram descritos e parte delas podem causar doenças em animais e humanos[4].

3.2.1 Flavivírus

Todos os vírus pertencentes ao gênero *Flavivírus* (família Flaviviridae) possuem RNA de fita simples e polaridade positiva com aproximadamente 11 kb. O genoma geralmente codifica um único quadro de leitura aberto (*Open Reading Frame)* que é flanqueada por regiões 5' e 3' não traduzidas (*Untraslated Regions*) de ~ 100 e ~ 400-700 nucleotídeos, respectivamente. Os *Flavivírus* mais conhecidos são transmitidos horizontalmente entre artrópodes hematófagos e hospedeiros vertebrados e possuem 40-50 nm de diâmetro [5,6–8]

Atualmente, entre os flavivírus que circulam entre hospedeiro vertebrado e mosquito com destaque para saúde pública estão dengue, febre amarela, Zika, vírus da encefalite japonesa, "*West Nile"* e "*Saint Louis"* [7,9].

3.2.2 Zika

O vírus Zika tem um histórico recente pois, foi descoberto em Uganda em meados de 1940 ficando limitado aos continentes Africano e Asiático nas décadas seguintes. Em 2007, o vírus passou a circular na Micronésia chegando a Polinésia Francesa entre 2013-2014 e então, atingiu a região da América Central e do Sul em 2015 e se disseminou para a América do Norte em 2016[10].

No início de 2015, o primeiro caso de Zika autócne foi diagnosticado no Brasil e no final desse mesmo ano já haviam pelo menos entre 400.000 - 1.300.000 casos suspeitos da doença que inicialmente parecia ser caracterizada como exantemática, mas de forma surpreendente mostrou ligação com o aumento assustador do número de bebês com microcefalia (onde grande parte das mães foram diagnosticadas com Zika durante a gestação)[11].

Desta forma e por conta da situação apresentada acima, o Ministério da Saúde, através da Portaria Número 1.813 de 11 de Novembro de 2015 declarou situação de Emergência em Saúde Pública de Importância Nacional (ESPIN)[12].

Outras complicações associadas a essa doença viral foram surgindo principalmente de cunho neurológico como a síndrome de Guillain Barré (SGB). A síndrome após infecção por ZIKV foi observada na Polinésia Francesa, onde uma relação de tempo foi estabelecida entre os dois, seguida de confirmação biológica. Casos de SGB causados pelo ZIKV também foram observados no Brasil na cidade de Recife, sendo que o vírus foi detectado por RT-PCR no líquido cefalorraquidiano em oito pacientes com manifestações neurológicas[13].

Após esta descoberta no Brasil, a Polinésia fez análises retroativas e percebeu que a microcefalia também havia ocorrido na região e outras complicações neurológicas haviam sido detectadas quando essa região passou por um surto de Zika[14].

3.2.3 Dengue

A dengue é uma doença causada por um vírus de polaridade positiva, classificada em quatro sorotipos denominados de (DENV 1, DENV 2, DENV 3, DENV 4) sendo endêmica em mais de 100 países desde a Ásia, Américas, região Oeste do Pacífico, África, regiões do mediterrânio[15] (FIGURA 1).

Atualmente, aproximadamente 400.000.000 de pessoas são infectadas por dengue todos os anos sendo que 25% dos casos apresentam sinais clínicos desde leves até severos com risco de morte. Avaliando as regiões

com risco de infeção e o número de casos estima-se que pelo menos 3,9 bilhões de pessoas em 128 países estejam em áreas de risco[16,17].

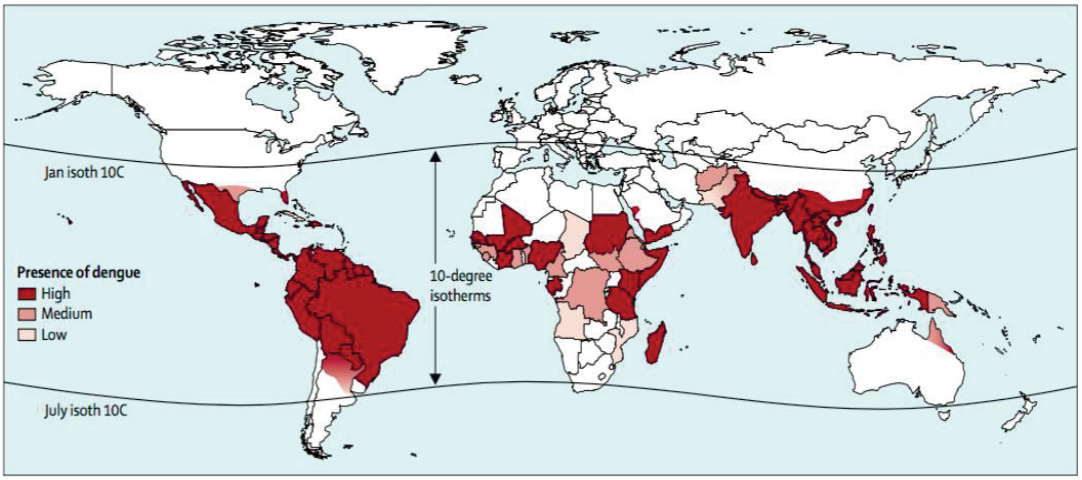

FIGURA 1 - SURTO GLOBAL DE DENGUE EM 2014
FONTE: [15]

A doença causada pelos vírus da dengue apresenta normalmente sintomas semelhantes aos da gripe como dor de cabeça, porém a gripe, além de causar coriza pode também desencadear problemas respiratórios como: apnéia e dispnéia. Os principais sinais e sintomas são febre alta que pode estar acompanhada por dor de cabeça, dor retro-ocular, náusea e vômito, *rash*. Além disso, o quadro pode tornar-se mais grave e o paciente ser acometido por dengue severa (febre hemorrágica) onde, atenção especial deve ser dada aos sinais de alerta como dor abdominal intensa, vômito persistente, sangramento nas gengivas, fadiga e ainda pode levar ao choque com sangramento severo e comprometimento grave de órgãos do paciente[13].

3.2.4 Família Togaviridae

A família Togaviridae é composta pelos gêneros: Rubivirus e Alfavirus. Esses vírus contém um genoma de RNA de cadeia simples com polaridade positiva com aproximadamente 11-12 kb e um diâmetro de 60-70 nm. Os principais vírus são responsáveis por diversos tipos de encefalites e síndromes febris[5,18]

A maioria dos alfavírus completam seu ciclo em artrópodes hematófagos podendo causar doenças em animais e humanos. Neste gênero podemos incluir como exemplo o vírus chikungunya, o vírus *Ross River*, vírus *Sindbis*, vírus da encefalite equina Venezuelana e o vírus Mayaro[5,18,19].

3.2.5 Chikungunya

Chikungunya é um vírus originário da África, e a febre chikungunya é considerada uma doença grave e debilitante sendo transmitido ao homem preferencialmente pela picada do mosquito *Aedes aegypti*. O vírus chikungunya de acordo com o idioma bantu faz referência a posição curvada do paciente por conta de intensas dores nas articulações além disso, o mesmo pode levar a febre alta e erupções cutânea impedindo muitas vezes que o paciente desenvolva suas atividades diárias e em muitos casos promover a incapacidade temporária. Essa infecçao tem deixado um rastro de adultos e idosos com dores crônicas graves que pode sobrecarregar a demanda do Sistema Único de Saúde [20,21].

No Brasil, o vírus chikungunya teve entrada recente e o primeiro caso de chikungunya foi diagnosticado em 2014 e o genótipo inicial foi o asiático, diagnosticado no Estado do Amapá (AP). No mesmo ano ocorreu uma segunda entrada de um segundo genótipo proveniente da África do Sul o qual foi introduzido também em 2014 em Feira de Santana, no Estado da Bahia (BA) [21,22]

3.2.6 Vetores de Zika, dengue e chikungunya

Os artrópodes foram descobertos como parte de ciclo biológicos de doenças que envolvem os humanos. Entre os agentes etiológicos que são transmitidos por artrópodes infectados podemos citar vírus, bactérias, protozoários e helmintos[23].

Os vírus estudados nesta tese têm como vetor principal o mosquito *Aedes aegypti* e ainda podem ser transmitidos por outras espécies de *Aedes sp.* e entre as outras espécies deste gênero podemos citar com um grau de importância o *Aedes albopictus*.

Atualmente a distribuição global de *Aedes aegypti* (FIGURA 2) e *Aedes albopictus* (FIGURA 3) mostram que estas espécies estão presentes em todos os continentes fortalecendo a dificuldade da eliminação de doenças associadas aos flavivírus e alfavírus, sendo que cerca da metade da população mundial corre o risco de uma infecção[24].

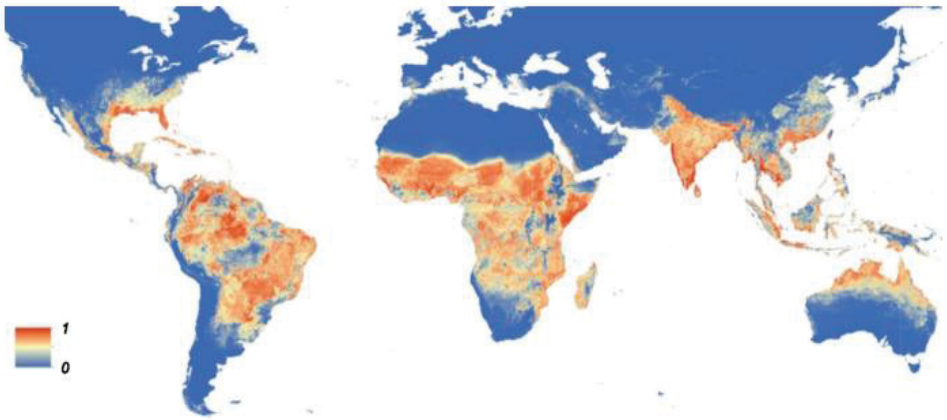

FIGURA 2 - MAPA GLOBAL DA DISTRIBUIÇÃO PREVISTA DE *Aedes aegypti*.
FONTE: O mapa descreve a ocorrência de *Aedes aegypti* (Azul = 0 e vermelho =1)[24].

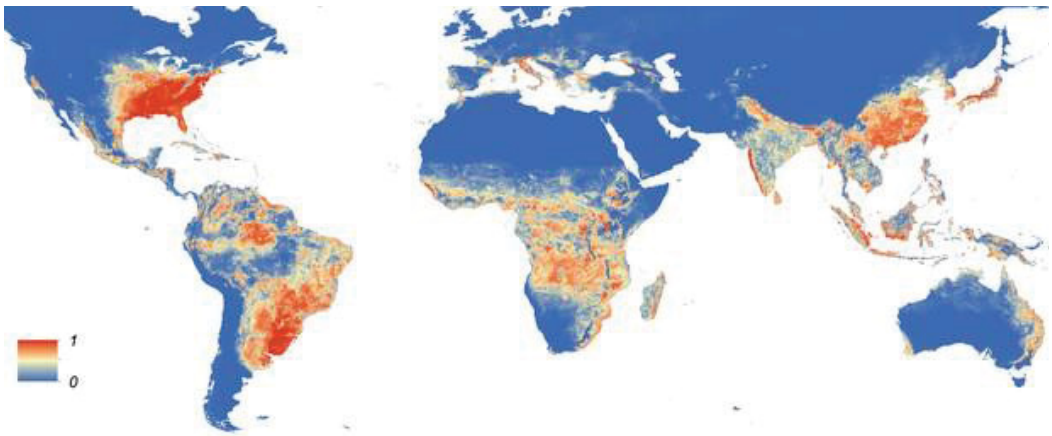

FIGURA 3 - MAPA GLOBAL DA DISTRIBUIÇÃO DE *Aedes albopictus*.
FONTE: O mapa descreve a ocorrência de *Aedes albopictus* (Azul = 0 e vermelho =1)[24].

A FIGURA 4 mostra uma fêmea de *Aedes aegypti* durante seu repasto sanguíneo onde suas principais características estão ressaltadas como a cor que pode variar de marrom a preto, manchas brancas pelo corpo e anéis brancos nas pernas e antenas pilosas [25].

FIGURA 4 - *Aedes aegypti*
FONTE: http://www.who.int/en/ 05/01/2018 às 13:53 hs.[25]

3.3 OUTRAS FORMAS DE TRANSMISSÃO DA INFECÇÃO POR ARBOVÍRUS

Em muitos casos os pacientes infectados por arboviroses são considerados assintomáticos, não permitindo diagnósticos clínicos apropriados mas, podendo ser fontes de contaminação para outras fêmeas de mosquitos, ou ainda para bolsas de sangue durante a doação[26] e outras pessoas durante relações sexuais[27,28], no leite materno[26,28] e transmissão vertical.[28]

3.4 DIAGNÓSTICO CLÍNICO DE ARBOVÍRUS

Como citado no item acima grande parte dos casos de arboviroses são assintomáticos e quando os pacientes apresentam sinais e sintomas relacionados aos três arbovirus ocorre um grande problema no diagnóstico clínico.

Quando os doentes apresentam sinais e sintomas os mesmos são muito semelhantes com a presença de alguns e/ou todos os sinais e sintomas; febre, *rash,* prurido, conjuntivites, dores de cabeça, dores retro-oculares; tanto nos casos sintomáticos de dengue, Zika e chikungunya[29] conforme QUADRO 1.

Por conta destes problemas, onde os principais sinais e sintomas dos pacientes são similares é importante realizar testes laboratoriais

principalmente em áreas endêmicas e áreas onde existe co-circulação de arbovírus[30].

QUADRO 1- PRINCIPAIS SINTOMAS DE DENGUE, CHIKUNGUNYA E ZIKA.

	DENGUE	CHIKUNGUNYA	ZIKA
PRINCIPAIS SINTOMAS			
FEBRE	Sempre presente: alta e de início imediato	Quase sempre presente: alta e de início imediato	Pode estar presente: baixa
ARTRALGIA (DORES NAS ARTICULAÇÕES)	Quase sempre presente: dores moderadas	Presente em 90% dos casos: dores intensas	Pode estar presente: dores leves
RASH CUTÂNEO (MANCHAS VERMELHAS NA PELE)	Pode estar presente	Pode estar presente: se manifesta nas primeiras 48 horas (normalmente a partir do 2º dia)	Quase sempre presente: se manifesta nas primeiras 24 horas
PRURIDO (COCEIRA)	Pode estar presente: leve	Presente em 50 a 80% dos casos: leve	Pode estar presente: de leve a intensa
VERMELHIDÃO NOS OLHOS	Não está presente	Pode estar presente	Pode estar presente

FONTE: https://agencia.fiocruz.br/zika-chikungunya-e-dengue-entenda-diferen%C3%A7as

3.5 DIAGNÓSTICO LABORATORIAL DE ARBOVÍRUS

O laboratório atualmente é um local decisivo para o diagnóstico de vírus complementando os achados clínicos, pois através dos testes diagnósticos é possível identificar o vírus responsável pelo quadro clínico dos arbovírus estudados.

No laboratório os testes mais usados baseiam-se em culturas virais, testes sorológicos para detecção de antígenos e anticorpos, testes moleculares para detecção de ácidos nucleicos virais e testes histológicos e citológicos. Atualmente, um grande número de testes moleculares e sorológicos estão sendo desenvolvidos e são mais rápidos facilitando assim o diagnóstico clínico para a maioria dos vírus. A cultura tem perdido notoriedade pois, as mesmas são mais demoradas e caras[31]. Os testes sorológicos podem apresentar alto risco de reações cruzadas, enquanto que os testes moleculares apresentam alta sensibilidade e especificidade. Na TABELA 1 estão descritos os principais métodos para a identificação viral.

TABELA 1 - TÉCNICAS UTILIZADAS EM DIAGNÓSTICO VIROLÓGICO

Cultura de células
Testes sorológicos
Testes moleculares
Microscopia eletrônica
Citologia
Histologia

FONTE: *Adaptado de "Special Selection: Medical Microbiology Diagnostic virology Rationale for Specific viral Diagnosis"* [31].

Os testes mais confiáveis para arbovírus são os testes NAT e ELISA para a glicoproteína não estrutural (NS1) que detecta infecções em fase aguda. A sorologia pode ser prejudicada por conta de vacinas virais como febre amarela, dengue e encefalite japonesa. Desta forma existe uma necessidade crucial para desenvolvimento de testes multiplex que sejam capazes de realizar o diagnóstico diferencial dessas arboviroses. A RT-qPCR está entre os principais testes usados no diagnóstico de arbovírus e é a metodologia que foi usada para o kit Biomol ZDC[32].

3.5.1 Real Time PCR

A reação em cadeia da polimerase é uma ferramenta que tornou a vida dos cientistas bem mais fácil. A definição do processo é de uma reação cíclica onde os passos se dividem em separação da dupla fita de DNA (desnaturação), o anelamento dos oligonucleotídeos específicos às suas regiões correspondentes e, finalmente, ocorre a extensão e em cada ciclo o número de cópias de DNA é duplicado, ou seja, a reação é exponencial. Quando há a presença de um alvo, por exemplo, em 40 ciclos pode-se chegar a gerar 2^{40} fragmentos específicos, caso a reação tenha 100% de eficiência[33]. A reação ocorre na presença de um DNA alvo específico, oligonucleotideos, íons de magnésio, nucleotídeos e uma enzima do tipo DNA polimerase[34].

Em PCR quando o material a ser detectado é o RNA ocorre um passo preliminar usando a transcriptase reversa (RT). A descoberta da RT aconteceu em meados de 1970 mostrando que essa enzima é uma DNA

polimerase dependente de RNA e que catalisa a síntese de uma fita de DNA complementar usando RNA como molde. O produto final é conhecido como DNA complementar (cDNA), um produto que não é sujeito à degradação por RNases, sendo assim mais estável que o RNA. Em reações de RT PCR após o passo de transcrição reversa o cDNA gerado é molde para a DNA dupla fita e o processo acontece como em uma reação comum de PCR. Essas reações são bastante usadas em diagnóstico de vírus que possuem como material genético RNA e também para a análise de expressão gênica[35].

É importante salientar que para que o PCR tenha qualidade alguns parâmetros como obtenção da amostra e estocagem, extração de ácidos nucleicos, pureza e qualidade dos ácidos nucleicos, desenho dos oligonucleotídeos, validação dos oligonucleotídeos, validação do gene de referência devem ser considerados[36].

Já em 1991, a tecnologia de amplificação de DNA era considerada uma estratégia e muito disso estava ligado a uma enzima termoestável isolada de "*Thermus aquaticus"* que ficou conhecida como TAQ DNA polimerase[37]. Holland e colaboradores em 1991[38] aprofundando os trabalhos com PCR demonstraram que a enzima TAQ apresentava a atividade 5'-3' exonuclease usando além de oligonucleotídeos sondas marcadas com isótopos radioativos. Nesse trabalho há uma ilustração típica mostrando como o processo ocorre, e que é citada com frequência, conforme FIGURA 5.[38]

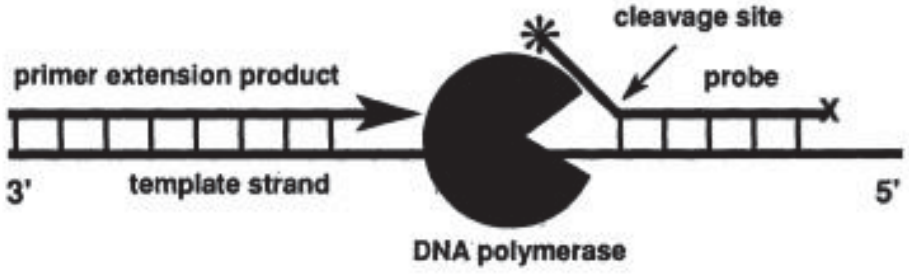

FIGURA 5 - DETECÇÃO DE PRODUTOS DE PCR COM BASE NA ATIVIDADE 5'-3' DA TAQ POLIMERASE.
FONTE: [38]

O início das reações de PCR em tempo real pode ser determinado quando se acoplou a câmera para captar a fluorescência de um intercalante

de DNA, o brometo de etídeo, aumentando a sensibilidade e especificidade do processo podendo inclusive iniciar a quantificação de amostras[39].

Durante os primeiros ciclos do PCR em tempo real é difícil distinguir a fluorescência liberada pelo anelamento da sonda ao alvo específico do *background* (ruído), entretanto, com o passar dos ciclos esse sinal se estabiliza (caso o DNA alvo esteja presente) e a fluorescência seja emitida exponencialmente até um ponto em que a reação atinge um platô seja por escassez de oligonucleotídeos, sondas, enzima ou qualquer outro componente da reação.

Então, a PCR em tempo real pode ser dividida em 3 fases inicial, logarítmica e platô como está demonstrado na FIGURA 6.[34]

As amostras que possuem maior quantidade de material genético atravessam o *threshold*, determinado pelo pesquisador manualmente e/ou pelo equipamento e que deve estar na fase logarítmica da reação, antes daquelas que possuem menor quantidade de material. Dessa forma as reações são designadas como inversamente proporcionais. Quanto maior o *Cycle threshold* (Ct), menor quantidade de material, e o inverso, quanto maior a quantidade de material menor Ct, isso é bastante importante para a interpretação dos resultados demonstrado abaixo na FIGURA 6.[34]

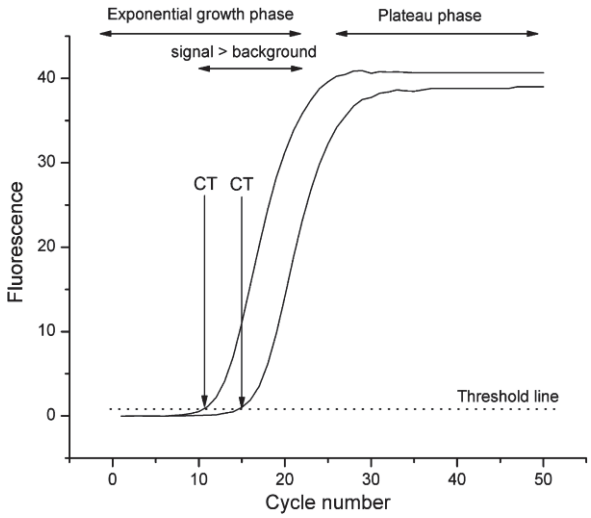

FIGURA 6 - CURVAS DE RESPOSTA DE PC REM TEMPO REAL.
FONTE: [34]

O uso de radioatividade e brometo de etídeo foram descontinuados no processo de PCR em tempo real e temos diferentes *reporters* que liberam fluorescência. Como exemplo pode-se citar as sondas de hidrólise, SYBR GREEN, molecular beacons e scorpions[40,34]. O uso de sondas oferece grande vantagem por conta da possibilidade de desenvolvimento de ensaios multiplex, como os usados nesta tese.

A tecnologia de real time PCR confirmou através de seu desenvolvimento e versalidade avanços em diferentes áreas como biomedicina, microbiologia, veterinária, agricultura, farmacologia, biotecnologia e toxicologia. Muitos testes estão descritos na literatura e também são usados para diagnóstico clinico e o crescimento das publicações na área é exponencial, sendo que em 12 de abril de 2018 o banco de artigos do '*PubMed"* demonstrou usando como palavra de busca "*REAL TIME PCR*" mais de 150 mil artigos publicados. Usando PCR tanto para o diagnóstico de vírus, bactérias, protozoários, fungos, organismos geneticamente modificados, sendo portanto uma ferramenta bastante importante principalmente por minimizar o tempo gasto frente a outras técnicas [41,40].

Os testes de PCR em tempo real, mesmo apresentando alta sensibilidade e especificidade, precisam ser validados através de controles.

3.6 CONTROLE PARA VALIDAÇÃO DE TESTES DIAGNÓSTICOS

Atualmente, os testes moleculares para doenças infecciosas têm sido essenciais na área clínica para orientar os médicos nas condutas terapêuticas a serem adotadas no início e durante o tratamento de pacientes. Entre estes métodos, a reações de PCR em tempo real e a RT-PCR em tempo real têm sido muito utilizadas devido a sua alta sensibilidade e especificidade na detecção de ácidos nucleicos de bactérias, fungos e vírus[42].

Assim, a padronização inequívoca da metodologia e a implementação de controles que possam garantir todos os processos laboratoriais, são realizados desde o início da manipulação de uma amostra clínica de paciente até o momento da liberação do resultado final do teste realizado, são imprescindíveis. Os controles positivos são representados por uma sequência

de DNA ou RNA diferente ou similares à sequência do ácido nucleico que está sendo pesquisada e o seu uso tem por objetivo assegurar a detecção do patógeno, reduzindo a possibilidade do produto diagnóstico gerar resultados falso - negativos[43].

Os resultados falso-negativos podem ser decorrentes de fatores como erros na extração da amostra, mal funcionamento do termociclador, falhas dos operadores, falhas no teste devido a inibição da PCR ou RT- PCR. A maneira mais comum de detectar inibidores nestas reações de amplificação é a inclusão de um controle interno positivo em cada amostra a ser analisada. Assim, este controle é extraído simultaneamente à amostra clínica e é amplificado no mesmo tubo em que está sendo feita a pesquisa do patógeno alvo[44,45]

Em um produto diagnóstico o controle interno deve ser sempre combinado com um controle positivo externo para provar a funcionalidade da mistura de reação para amplificar o patógeno alvo[46]. Esta combinação exclui a existência de inibição e de outros possíveis erros e assegura que um resultado negativo seja verdadeiro e não seja uma consequência de falhas em algum dos procedimentos.

Como controles internos podem ser utilizados genes endógenos ou exógenos. Os genes endógenos ou "*housekeeping genes*" estão presentes no genoma de diferentes espécies, são expressos por diferentes tipos celulares e são utilizados no diagnóstico molecular para verificar se as condições de PCR são ótimas[47]. A amplificação de um gene endógeno na reação assegura a origem e a qualidade da amostra de DNA ou RNA, d*a* mistura de reação e dos reagentes utilizados na sua amplificação porém, apresentam a desvantagem de ser amplificados utilizando oligonucleotídeos diferentes dos utilizados na amplificação do alvo (patógeno)[47].

Os principais genes endógenos descritos na literatura que têm sido utilizados como controle nas reações de PCR em tempo real são o gene da proteína *glyceraldehyde-3-phosphate desidrogenase* (GAPDH), β-actina, proteínas ribossomais, ubiquitina (UBQ), β-tubulina, proteína do RNA ribossomal 18S (18S rRNA) e fosfoglicerato Kinase (PGK)[48].

Embora o produto destes genes sejam constitutivamente expressos, existem variações nos níveis de expressão de seu RNA mensageiro em certas condições experimentais e por esta razão é crucial a sua validação para cada modelo particular de reação[49]. Estes controles são amplificados utilizando oligonucleotídeos e sondas desenhados baseando-se nas sequências nucleotídicas do gene escolhido como controle e a sua amplificação na reação de PCR precisa ser otimizada em multiplex com a sequência do alvo que está sendo pesquisada.

Os controles internos exógenos são adicionados as amostras clínicas e podem ter sequências nucleotídicas diferentes do alvo, sendo amplificados e detectados com oligonucleotídeos específicos para as suas sequências ou podem compartilhar sequências com o alvo sendo co-amplificados simultaneamente a estes, utilizando os mesmos oligonucleotídeos[50]. Porém, é necessário que seja adicionado na reação de amplificação uma sonda específica que possa distinguir o controle do microorganismo que está sendo pesquisado.

Os controles positivos são exógenos e o seu DNA ou RNA é extraído paralelamente as amostras clínicas em teste e no momento da reação de PCR são amplificadas em outro poço de reação. Da mesma forma que os controles internos podem ter ou não sequências específicas do alvo.

Plasmídeos construídos com sequências específicas do alvo têm sido preparados para diversos microorganismos e podem ser utilizados como controles internos ou como controles positivos[51–53] A desvantagem da sua utilização refere-se ao fato de que os plasmídeos são moléculas de DNA e por isso não tem a propriedade de controlar a fase de extração e de transcrição reversa que é necessária para amplificar vírus com genoma de RNA. Por esta razão outras estratégias de construção de controles têm sido propostas e estão descritas na literatura [54].

Uma destas estratégias propõe a utilização de partículas virais com propriedade de mimetizar os alvos que estão sendo pesquisados, ou seja, que apresentem um capsídeo e material genético protegido tendo assim a propriedade de controlar o processo de extração viral e da transcrição

reversa. Esta linha de controles está representada pelos vírus animais e pelos bacteriófagos.

Diversos artigos também se reportam a utilização de vírus animais. O vírus da febre bovina e o *Phocine distenper* vírus que foram utilizado como controle em reações de RT - PCR para o vírus da hepatite C[54,55].

Entre os bacteriófagos, o MS2 foi utilizado como controle em diversos métodos moleculares[56–58], e também o T4[59].

O controle *armored* estabelecido pela empresa Assuragen®, são partículas compostas pela proteína do envoltório do bacteriófago MS2 encapsidando um RNA exógeno. Estas partículas foram produzidas através de clonagem em plasmídeos e expressão e são resistentes à degradação por RNases e foram criadas inicialmente para o vírus HIV[60] e da Hepatite C[61]. Controles armored para diferentes patógenos estão descritos em artigos[62].

Em outra estratégia para estabelecer controles derivados do bacteriófago MS2 foram construídos dois vetores. No primeiro foi colocado o genoma inteiro do MS2 e no segundo foi inserido o genoma do MS2 modificado pela presença de um gene sintético no gene da replicase. Estes vetores foram utilizados para produzir expressar respectivamente partículas do MS2 e partículas recombinantes do MS2. Ambas foram avaliadas em um teste quantitativo para a hepatite C[63].

Transcritos sintéticos de RNA também têm sido utilizados como controles em diversos métodos moleculares[64]**.**

Portanto diversos modelos de controles já foram estabelecidos utilizando diversas estratégias e estão descritos na literatura e podem ser utilizados como modelo para a construção de controles para testes diagnósticos de outros microorganismos.

3.7 MS2

Os bacteriófagos são vírus de ocorrência natural que infectam somente as bactérias. Estes vírus foram descobertos por Frederick Twort em 1915 e por Felix d'Herelle em 1917 e desde está época continuam sendo estudados.

Os fagos são basicamente formados por uma molécula de ácido nucleico envolta por um capsídeo que é constituído por proteínas codificadas pelo genoma. Este capsídeo tem o papel importante de proteger o genoma da danificação de fatores ambientais e assegurar a liberação do mesmo nas células alvo (FIGURA 7) [65].

O bacteriófago MS2 pertence à família Leviviridae e tem um genoma de RNA de fita simples, polaridade positiva e um capsídeo com estrutura icosaédrica de 275 A°, não possui cauda e não possui apêndice de superfície. Cada partícula deste fago é constituída por 180 unidades monoméricas da proteína de envoltório, uma cópia da proteína de maturação (maturase) e uma única cópia de RNA genômico. Pelo fato de consistir de uma molécula de RNA e o MS2 ter um capsídeo a sua presença no final de uma reação garante o controle da descapsulação do RNA viral durante o processo de extração e da fase de transcrição reversa. Como o RNA é suscetível à degradação e inibição por RNases, o MS2 é um controle interno ou externo com a capacidade de monitorar desde o processo de extração até a amplificação, e pode ser usado como controle universal para testes moleculares em que se pretende detectar vírus que possuem como material genético RNA[63,65].

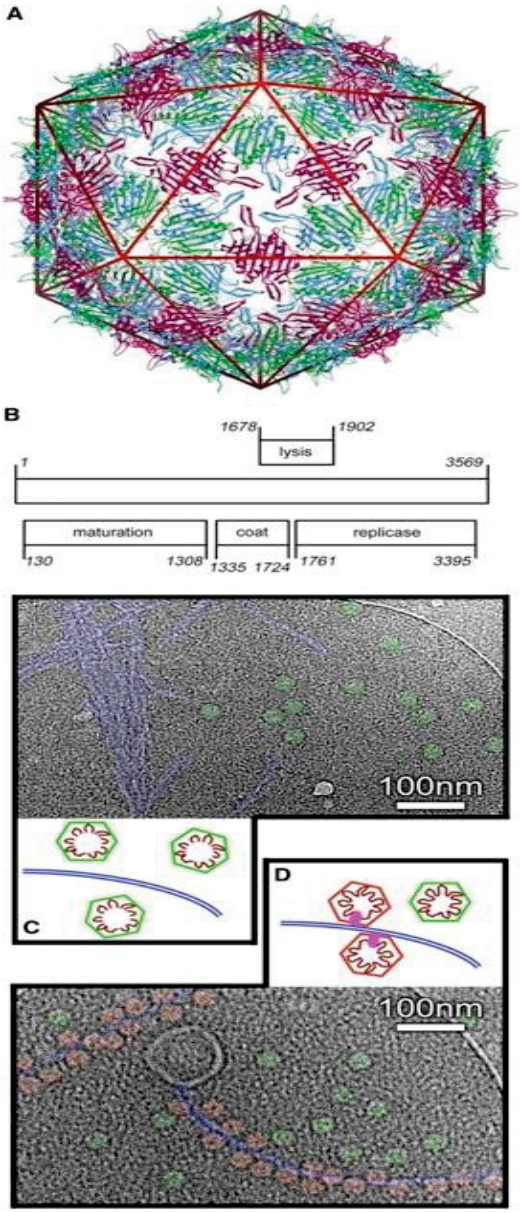

FIGURA 7-
A- ESTRUTURA DO CAPSÍDEO DO BACTERIÓFAGO MS2
B- ESTRUTURA DO GENOMA
C- MICROSCOPIA ELETRÔNICA DE VIRAL LIKE PARTICLES MS2
D- MICROSCOPIA ELETRÔNICA DE MS2 NÃO MODIFICADO
FONTE: [65]

3.8 KIT BIOMOL ZDC

Os testes realizados com o Kit BIOMOL ZDC são baseados na técnica da reação em cadeia da polimerase em tempo real com transcrição reversa (RT-qPCR). A qPCR permite a quantificação de sequências específicas de DNA ou RNA em uma amostra a partir de medidas de intensidade de fluorescência durante o andamento da reação. Na técnica de RT-qPCR,

ocorre inicialmente uma transcrição reversa (geração de cDNA a partir do RNA da amostra) seguida pela reação em cadeia da polimerase em tempo real (qPCR).

O Kit BIOMOL ZDC permite a identificação dos vírus Zika, Dengue (sorotipos 1 a 4) e Chikungunya, além do Controle Interno (CI) da reação. A detecção da presença de ácidos nucleicos dos patógenos e do controle Interno é feita pelo uso de sondas (oligonucleotídeos marcados com fluorescência) específicas para cada alvo molecular. O teste para cada patógeno é realizado em uma reação multiplex, onde existem reagentes específicos para o alvo do patógeno e para o Controle Interno; desta forma, o kit apresenta quatro reações multiplex distintas, sendo elas: Zika/CI, Dengue 1/Dengue 4/CI, Dengue 2/Dengue 3/CI e Chikungunya/CI.

A amplificação do Controle interno indica um funcionamento adequado da reação. Em resultados negativos, apenas o Controle Interno é detectado. Caso esta amplificação não seja detectada, a amostra deve ser retestada. Já a amplificação de material genético de patógenos indica presença de RNA viral na amostra.

É importante avaliar cada tipo de reação multiplex separadamente. Este kit foi desenvolvido para a realização de análises de perfil qualitativo, ou seja, avalia-se a presença ou ausência de alvo molecular.

A técnica de RT- qPCR é a base desse estudo de diagnóstico dos seguintes vírus que possuem como material genético RNA: Zika, dengue (sorotipos 1-4) e Chikungunya, foram distribuídos na placa de PCR, conforme FIGURA 8.

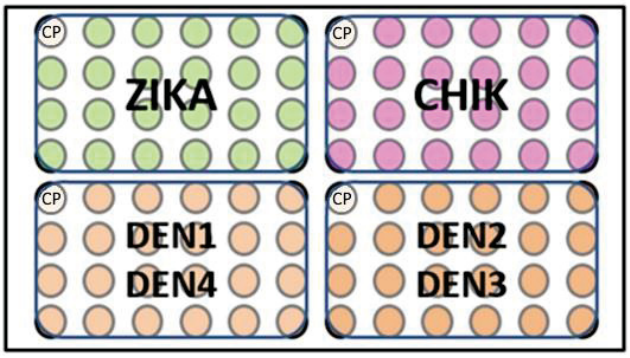

FIGURA 8 - PLACA DE PCR
FONTE: o autor (2018)

As 4 reações do kit do tipo *one step* RT-qPCR multiplex utilizando sondas de hidrólise estão descritas na Tabela 2.

TABELA 2 - KIT BIOMOL ZDC

Zika + controle Humano
Chikungunya + controle Humano
Dengue 1 + dengue 4 + controle Humano
Dengue 2 + dengue 3 + controle Humano

FONTE: o autor (2018)

O teste descrito acima é qualitativo, ou seja, permite detectar a presença ou ausência dos vírus e ainda garantir que o operador realizou de maneira correta o mesmo por conta da presença de controle interno humano e que o RNA apresentava boa qualidade, entretanto, esse kit apresentava uma grande lacuna, a ausência de um controle positivo.

O desenvolvimento de um controle positivo foi o objetivo dessa tese pois, é bastante laborioso trabalhar com culturas virais que seriam controles efetivos além disso, as culturas oferecem riscos, são mais caras e precisam de ambientes específicos e pessoal qualificado.

Desta forma, escolhemos desenvolver um controle derivado do MS2 para os 7 alvos presentes no kit pois, tem estrutura similar aos alvos que serão detectados pelo kit BIOMOL ZDC, RNA de fita simples polaridade positiva e capsídeo.

O nosso grupo já possuía expertise na técnica de produção de controles usando esse bacteriófago quando utilizado para a construção de um controle interno positivo para detecção do vírus da hepatite C.

4 RESULTADO E DISCUSSÃO

Diante do acima exposto nosso trabalho será apresentado na forma de dois capítulos: Artigos submetidos para revistas que abordam temas somente relacionados a vírus:

O primeiro artigo que demonstra todo o processo de construção e validação do controle positivo e o seu uso final para a validação do kit Biomol ZDC fechando a principal lacuna do mesmo. Neste artigo apresentamos o desenvolvimento do controle positivo baseado no bacteriófago MS2 para ser usado em ensaios para detectar o ácido nucleico do Zika, chikungunya e dos quatro sorotipos da dengue. O pET 47b(+) – MS2-ZDC foi construído após a inserção de uma sequência sintética em um único local BamHI (dentro do gene da replicase do genoma MS2) do pET47b(+) – MS2. Todas as construções foram confirmadas por sequenciamento. O pET27b(+) -MS2 -ZDC pode gerar sucesso partículas de MS2 -ZDC cujo genoma abriga a sequência sintética no gene da replicase, e como consequência uma enzima não funcional foi produzida, bloqueando a replicação do genoma.

Já no segundo artigo mostramos o controle positivo sendo usado em um estudo epidemiológico em Porto Velho, Rôndonia (RO), onde os primeiros casos de Zika foram detectados. Neste artigo o controle foi comparado com culturas positivas de vírus para garantir os resultados.

Amostras de RNA extraídas do soro de 164 pacientes foram testadas em um ensaio de RT-PCR em tempo real. As análises da amplificação revelaram que 7 (4,3%) das amostras deram positivas apenas para o RNA de ZIKV, e 157 deram negativas para todos os sorotipos de DENV, CHIK e RNA do ZIKV. Os resultados negativos do RT-PCR em tempo real foram validados pelo controle interno. Todas as amostras de RNA mostraram amplificação da sequência de controle interno (um transcrito humano endógeno), para garantir que essas amostras não fossem degradadas e que os resultados negativos fossem verdadeiramente negativos. O teste sorológico para o antígeno NS1 DENV também foi negativo para todas as amostras de soro.

A avaliação microscópica de 164 esfregaços de sangue periférico também foi negativa para a infecção por malária. Durante a anamnese, os

principais sinais e sintomas relatados por nossos pacientes que foram considerados relevantes para nosso estudo foram: conjuntivite 7/164 (4,3%), febre 84/164 (51,2%), cefaleia 78/164 (47,56%), calafrios 10/164 (6,1%), prurido 20/164 (12,2%), exantema 33/164 (20,1%), artralgia 58/164 (35,3%), mialgia 44/164 (26,8%) e retro- dor orbital 32/164 (19,5%).

Dos sinais e sintomas relatados pelos pacientes com viremia, apenas a conjuntivite teve relevância estatística (OR = 12,16, IC95%, 1,88-78,63, p = 0,03), com correlação positiva entre conjuntivite e positividade para ZIKV. Portanto, pacientes com infecções por ZIKV são mais propensos a desenvolver condições inflamatórias na região ocular ($p<0,05$) do que indivíduos não infectados.

Além disso, a especificidade do ensaio de RT-PCR em tempo real (kit Biomol ZDC) para ZIKV, DENV e CHIKV foi avaliada testando o ensaio com amostras reconhecidamente positivas para *Plasmodium falciparum*; Vírus Mayaro, febre amarela e febre Oropouche; e ZIKV, DENV1, DENV2, DENV3, DENV4 e CHIKV. Os dados mostraram somente amplificações específicas, confirmando a ausência de reatividade cruzada do ensaio com os patógenos avaliados. Os ensaios foram validados pelo controle positivo.

Em anexo encontram-se dois artigos publicados, referentes ao cultivo de microalgas, os quais foram escritos durante os dois primeiros anos do doutorado onde pesquisava o cultivo de microalgas em resíduos da agroindústria.

CAPÍTULO 1

EXTERNAL CONTROL VIRAL-LIKE PARTICLE CONSTRUCTION FOR DETECTION OF EMERGENT ARBOVIRUSES BY REAL TIME PCR

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Manuscript Draft

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Title: External control viral-like particle construction for detection of emergent arboviruses by real time PCR.

Article Type: Short communication

Keywords: RT-PCR, Arboviruses, Positive Control

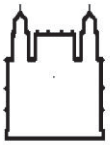
Corresponding Author: Dr. Rita de Cássia Pontello Rampazzo, Ph.D

Corresponding Author's Institution: Instituto de Biologia Molecular do Paraná

First Author: Ivo A Borghetti

Order of Authors: Ivo A Borghetti; Miriam R Zambenedetti; Luciana Requião; Marco A Krieger; Rita de Cássia Pontello Rampazzo, Ph.D

Cover Letter



FIOCRUZ
Fundação Oswaldo Cruz

Curitiba, Brazil
June 1st, 2018.

Dear Editorial Board of the Journal of Clinical Virology :

Please, find enclosed the manuscript by Borghetti *et al.* entitled "**External control viral-like particle construction for detection of emergent arboviruses by real time PCR**". We are submitting this manuscript for consideration as a Short Communication.

Our study describes the development of an external positive control for RT-PCR using a modified MS2 bacteriophage vector with seven targets from arboviruses that will be detected using the ZDC Biomol kit (Instituto de Biologia Molecular do Paraná). The ZDC Biomol kit is able to detect Zika, chikungunya, all dengue serotypes, and an internal control human transcript in 4 multiplex reactions. The positive control derived from the MS2 vector can successfully be used in conjunction with the multiplex reactions described above. Furthermore, by integrating this control into the ZDC Biomol assay, we can monitor all steps of the diagnostic process and ensure the quality of the results.

Our current report has not been and will not be submitted for publication to any other scientific journal. All authors agree with the final text and declare that they have neither financial nor nonfinancial competing interests. On behalf of all the authors I wish to thank you in advance for your care and attention in handling this Short Communication.

Yours sincerely,

Dr Rita de Cássia Pontello Rampazzo

Instituto de Biologia Molecular do Paraná

Rua Prof. Algacyr Munhoz Mader 3775 - Curitiba – PR - 81350-010 - Brasil

Tel: (55-41) 3316-3260

Conflicts of Interest Statements

External control viral-like particle construction for detection of emergent arboviruses by real time PCR.

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from rcprampazzo@gmail.com

The authors declare no potential conflicts of interest.

Ivo Alberto Borghetti Ivo A Borghetti

Miriam Ribas Zambenedetti Miriam Ribas Zambenedetti

Luciana Requião Luciana Requião

Marco Aurélio Krieger

Rita de Cássia Pontello Rampazzo Rita de Cássia Pontello Rampazzo

***Title Page (including author details, affiliations and word count)**

External control viral-like particle construction for detection of emergent arboviruses by real time PCR.

Ivo Alberto Borghetti[1,2#], Miriam Ribas Zambenedetti[1#], Luciana Requião[1], Marco Aurélio Krieger[1,2,3], Rita de Cássia Pontello Rampazzo[1*]

[1]IBMP- Instituto de Biologia Molecular do Paraná, Curitiba, PR, Brazil.

[2] Universidade Federal do Paraná, Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Curitiba, PR, Brazil.

[3]Instituto Carlos Chagas-ICC/FIOCRUZ, Curitiba, PR, Brazil.

# authors contributed equally

*Corresponding author: rcprampazzo@gmail.com, rirampazzo@ibmp.org.br

IBMP- Instituto de Biologia Molecular do Paraná, Curitiba, PR, Brazil

Rua Professor Algacyr Munhoz Mader, 3775,

Cidade Industrial - Curitiba - PR

Brazil – 81350-010

Tel.: +55- 41- 33163260

Fax: +55- 41- 33163260

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Ivo Alberto Borghetti[1,2#], Miriam Ribas Zambenedetti[1#], Luciana Requião[1], Marco Aurélio Krieger[1,2,3], Rita de Cássia Pontello Rampazzo[1*]

[1]IBMP- Instituto de Biologia Molecular do Paraná, Curitiba, PR, Brazil.

[2] Universidade Federal do Paraná, Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Curitiba, PR, Brazil.

[3]Instituto Carlos Chagas-ICC/FIOCRUZ, Curitiba, PR, Brazil.

# authors contributed equally

*Corresponding author: rcprampazzo@gmail.com, rirampazzo@ibmp.org.br

IBMP- Instituto de Biologia Molecular do Paraná, Curitiba, PR, Brazil

Rua Professor Algacyr Munhoz Mader, 3775,

Cidade Industrial - Curitiba - PR

Brazil – 81350-010

Tel.: +55- 41- 33163260

Fax: +55- 41- 33163260

ABSTRACT

Background: Arboviruses have been emerging and reemerging worldwide, predominantly in tropical and subtropical areas. As many arbovirus infections, including dengue (DENV), Zika (ZIKV), and chikungunya (CHIKV) have similar signs and symptoms so, clinical diagnosis of arbovirus infections is challenging. Therefore, reliable laboratory tests are necessary to improve the clinical management of patients with suspected arbovirus infections. Real-time reverse-transcription PCR (RT-PCR) is among the more effective methods to distinguish these viruses.

Objective: The aim of this study was to construct a unique positive external control using genetic engineering for specific use in RT-PCR assays to detect Zika, dengue, and chikungunya.

Study design: An external control derived from the MS2 bacteriophage was constructed using sequences from arbovirus and human genomes. Laboratories were asked to test the control in the ZDC Biomol kit, a RT-PCR kit which is able to detect Zika, dengue serotypes 1-4, chikungunya, and an internal human control.

Results: RNA extracted from the external control was able to be amplified and detected in RT-PCR assays for each virus detected by the ZDC Biomol kit. The external control, samples from viral culture, and infected patient samples display similar amplification using this assay.

Conclusion: The pET47b(+)MS2-ZDC vector is a viable expression system for the production of external control viral-like particles (MS2-ZDC). The RNA from

45 the recombinant particles can be easily extracted and incorporated into the ZDC
46 Biomol kit. Thus, the MS2-ZDC particle is biosafety and, effective positive
47 control for use in the ZDC Biomol kit.

48 **Keywords:** RT PCR, Arboviruses, Positive Control

49

INTRODUCTION

Emerging and reemerging viruses transmitted by arthropod vectors, such as yellow fever, Zika, dengue, chikungunya, Rift Valley fever virus, Japanese encephalitis virus, West nile virus, Saint Louis encephalitis virus, Murray Valley encephalitis virus, Usutu virus, Spondweni virus, and O'nyong-nyong have transmission cycles in urban environments. These viruses have overlapping clinical symptoms, and many patients develop serious physiological manifestations that can include death in severe cases[1,2].

Since the clinical symptoms of arbovirus infections overlap, laboratory diagnostics are necessary to distinguish between them. Viral infections can be diagnosed through different methods including viral culture, serology, and molecular methods, and these same techniques are used to test for arbovirus infections[3,4] Real-time reverse-transcription polymerase chain reaction (RT-PCR) has been used to detect dengue (a virus that is part of family *Flaviviridae*, genus *Flavivirus*), Zika (a virus that is part of family *Flaviviridae*, genus *Flavivirus*) and, chikungunya (a virus that is part of family *Togaviridae*, genus *Alphavirus*) worldwide in different samples such as serum, urine, cerebrospinal fluid, and saliva. RT-PCR is easier to conduct than the other methods used to detect pathogens, and its quality depends specifically on the samples, the human operator, the nucleic acid test (NAT) kit, and PCR equipment[5–8].

Our group developed a Nucleic Acid Test (NAT) for diagnosis of Zika, dengue serotypes 1, 2, 3, and 4, and chikungunya with an internal human control. This kit was used as a tool for Zika detection in saliva and urine from

74 symptomatic patients. The results were validated with the extraction of RNA
75 from viral cultures, which is a laborious method[5]. Another group screened for
76 asymptomatic infection in 676 units of donated blood and discovered that there
77 were no positive samples in Juazeiro, Bahia, Brazil, during a Zika epidemic.
78 Sharma *et al.*,[9] also used RNA from viral cultures as positive controls. In this
79 article, we describe the development of a reliable positive control for this assay.
80
81

82

83 **OBJECTIVES**

84 In order to improve molecular diagnostics of Zika, dengue 1, 2, 3, and 4,
85 and chikungunya, the purpose of this study was to develop a unique external
86 control based in a modified MS2 bacteriophage vector. Additionally, we
87 demonstrate the applicability of this control to RT-PCR assays using the ZDC
88 Biomol kit.

89

STUDY DESIGN

External control construction

The synthetic nucleotide sequence was designed based on targets of the ZDC Biomol kit. Synthetic sequence for the specific targets were analyzed using tools from the National Center for Biotechnology Information (NCBI). After the synthetic sequence was synthesized, it was cloned into the pET47b(+)-MS2 vector (GeneScript). The derived vector is referred to as pET47b(+)-MS2-ZDC.

External control production

The pET47b(+)-MS2-ZDC vector was transformed into NiCo21(DE3) competent *Escherichia coli* in accordance with the manufacturer's instructions (New England Biolabs, USA). The protocol for expression and purification was described previously by Zambenedetti et al.,[10] with some modifications. The expression of pET47b(+)-MS2-ZDC was induced by the addition of 0.5 mM isopropyl-1-β-D-thiogalactoside. After purification, viral-like-particles were stored at -20 ℃.

Nucleic acid isolation

RNA was extracted from viral stocks obtained from infected cells and from MS2–ZDC controls (aliquots of 140 µL) using the QIAamp viral RNA mini kit (QIAGEN®, Hilden, Germany) in accordance with manufacturer's instructions. RNA was eluted at a final volume of 60 µL.

111 **Stability**

112 The RNA was extracted from MS2-ZDC particles stored at -20ºC for 0, 6, and
113 12 months.

114 **Viability**

115 RNA from MS2-ZDC particles submitted to stress conditions was extracted.
116 Before extraction, the particles were thawed and submitted to special
117 temperature conditions: 37℃ and 70℃ twice for 1 or 2 hours. The RNA from
118 these particles was then compared to RNA from particles which were not
119 submitted to stress conditions.

120 **Nucleic Acid Testing**

121 Amplification was performed using 9.5 µl of extracted ZDC-MS2 RNA and RNA
122 from arbovirus cultures as a template in a 20 µl final volume RT-PCR reaction
123 to detect ZIKV, CHIKV, and DENV (serotypes 1-4) and an internal control using
124 a 7500 Real-Time PCR Instrument (Applied Biosystems®) following the
125 manufacturer's instructions.

126

127

128

129 **RESULTS**

130 *MS2-ZDC production*

131 We developed a positive external control based on a MS2 bacteriophage vector
132 to be used in assays to detect the nucleic acids from Zika, chikungunya, and
133 dengue virus serotypes 1-4. The pET47b(+)-MS2-ZDC vector was constructed
134 after the insertion of a synthetic sequence into a unique BamHI site of
135 pET47b(+)-MS2, which falls within the replicase gene of the MS2 genome. All of
136 the constructs were confirmed by sequencing. pET47b(+)-MS2-ZDC could
137 successfully generate MS2-ZDC particles whose genome harbored the
138 synthetic sequence in the replicase gene. Consequently, a non-functional
139 enzyme was produced, blocking replication of the genome.

140

141 *MS2-ZDC Control in the ZDC Biomol Kit.*

142 The RT-PCR design for our experiments was composed of 4 different reactions
143 which were able to detect 1) Zika, 2) dengue serotypes 1 and 4, 3) dengue
144 serotypes 2 and 3, and 4) chikungunya, as well as a human transcript in each
145 reaction as an internal control. RNA was extracted from MS2-ZDC particles and
146 subsequently evaluated using the ZDC Biomol RT-PCR kit. The amplification
147 results were equivalent to those obtained using samples from patients positive
148 for each virus tested. The MS2-ZDC RNA control showed specific amplification
149 for all targets in each reaction of the ZDC Biomol kit and was expected in the
150 same well in which the internal control also was amplified. The MS2–ZDC
151 control amplifications are shown in Figure 1.

152

153 *MS2-ZDC particle stability*

154 To determine if the MS2-ZDC particles could be stored long-term and maintain
155 stability, RNA extracted from MS2-ZDC particles stored at -20ºC were assayed
156 for Zika, chikungunya, and dengue 1-4 using the ZDC Biomol Kit. Aliquots
157 stored for 6 or 12 months were compared with RNA from fresh particles which
158 had not been stored (Table 1), and the results demonstrate no significant loss
159 of Ct with prolonged storage. Additionally, the standard deviation was lower
160 than 1 Ct. Therefore, the MS2-ZDC control particles can be stored at -20ºC for
161 up to 12 months without compromising their performance.

162

163 To further test the stability of the MS2-ZDC particles, we subjected them to
164 temperature stress conditions as follows: 37ºC and 70ºC for 1 or 2 hours. When
165 the RNA was assayed by RT-PCR, no significant difference in Ct values was
166 seen when compared to particles stored -20ºC. Therefore, the particles are
167 quite stable, even under changing temperature conditions (data not shown).

168

169

170 **DISCUSSION**

171 RT- PCR was first introduced in the mid-1990s for use in infectious
172 disease diagnostics. The method had already been recognized, and many
173 businesses were invested in this field[11]. Even in 1990, a number of studies had
174 demonstrated the practicality of identifying viral pathogens in many clinical and
175 epidemiological conditions using multiplex PCR[12,13]. Soon after, organizations
176 recognized the need to ensure the quality of RT-PCR results. The RT-PCR
177 diagnosis kits available already use and recommend controls, with a number of
178 different options available for use described in the literature. The most common
179 controls are plasmids, phages, transcripts, animal viruses, and synthetic
180 sequences[10,14–18].

181 Our study was focused on creating a positive control for a RT-PCR-
182 based diagnostic assay that detects Zika, dengue serotypes 1-4, and
183 chikungunya. We proposed an external positive control based in the MS2
184 bacteriophage modified with an extrinsic sequence. Generally, this strategy has
185 been chosen when only one target is being assayed, but we developed our
186 control to cover all seven targets in one multiplex RT-PCR assay[10]. There are
187 MS2-based strategies using more than one target for digital PCR (4 targets),
188 but our positive control (7 targets) had the same success and the stability of our
189 control was maintained for 12 months[19].

190 In conclusion, our study demonstrated successful construction,
191 expression, and purification of a novel external control which can be integrated
192 into the ZDC Biomol kit. Controls such as the one described here are especially

193 important to ensure diagnostic RT-PCR quality in the face of emerging
194 outbreaks of Zika, dengue, and chikungunya. Furthermore, the development of
195 the control was cost effective and less time consuming than the use of viral
196 cultures. The results of this study demonstrate the sensitivity and specificity of
197 our control in RT-PCR assays.

198 Currently, many dangerous viruses are endemic and spreading
199 worldwide, and these viruses must be handled at biosafety levels 2, 3, or 4.
200 Accordingly, our strategy may represent a good choice for positive control
201 design to ensure operator and environmental safety, as MS2 controls do not
202 cause damage to humans [20].
203

204

205 **Conflict of interest**

206 The authors declare no potential conflicts of interest.

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208

209

210 **ACKNOWLEDGEMENTS**

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214

215

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Table

1

2 Table 1. **ZDC-Biomol PCR results from MS2-ZDC particles under different**

3 **storage conditions.**

Reactions	Targets	0 months	6 months	12 months	SD
Duplex	Zika	22.19	23.16	21.47	0.85
	Control	21.38	21.96	21.99	0.35
Duplex	Chik	21.86	22.64	22.40	0.40
	Control	21.38	21.69	21.96	0.29
Triplex	Den1	20.10	20.98	20.91	0.49
	Den4	20.13	21.33	21.03	0.62
	Control	20.93	22.22	21.75	0.65
Triplex	Den2	20.54	21.20	20.94	0.33
	Den3	22.83	23.05	23.29	0.23
	Control	21.23	22.01	21.93	0.43

4

Figure

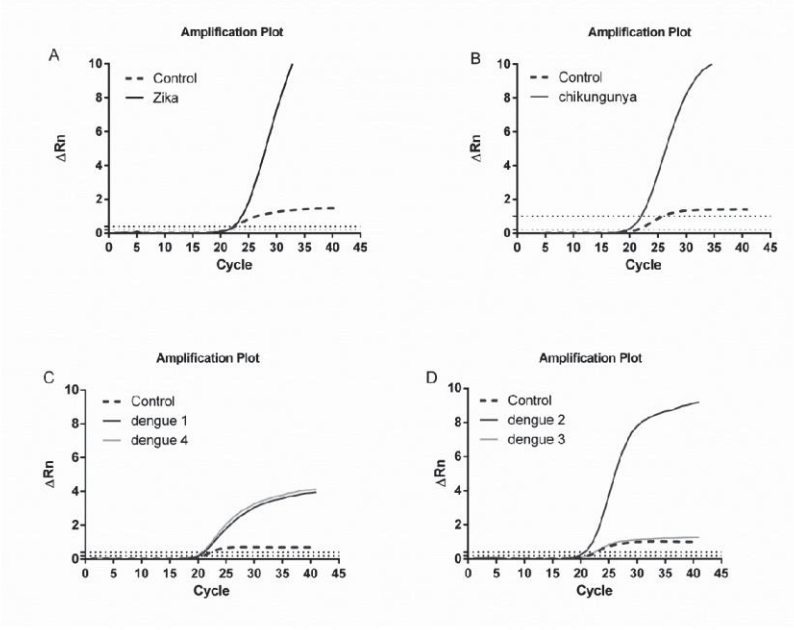

Figure 1. MS2-ZDC control RNA amplification plots in the ZDC Biomol Kit. MS2-ZDC control amplification in A) duplex Zika/internal control reaction, B) duplex Chikungunya/internal control reaction, C) triplex Dengue 1/Dengue 4/internal control reaction, and D) triplex Dengue 2/Dengue 3 /internal control reaction.

CAPÍTULO 2

EPIDEMIOLOGICAL PROFILEOF ZIKA, DENGUE, AND CHIKUNGUNYA VIRUS INFECTIONS IDENTIFIED BY MEDICAL AND MOLECULAR EVALUATIONS IN THE WESTERN BRAZILIAN AMAZON

Journal of Medical Virology

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EPIDEMIOLOGICAL PROFILE OF ZIKA, DENGUE, AND CHIKUNGUNYA VIRUS INFECTIONS IDENTIFIED BY MEDICAL AND MOLECULAR EVALUATIONS IN THE WESTERN BRAZILIAN AMAZON

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Complete List of Authors:	Vieira, Deusilene; Fundação Oswaldo Cruz; Universidade Federal de Rondonia; Centro de Pesquisa em Medicina Tropical Lopes, Debora; Fundação Oswaldo Cruz de Oliveira, Lucas; Fundação Oswaldo Cruz Zambenedetti, Miram; Instituto de Biologia Molecular do Paraná Requiao, Luciana; Instituto de Biologia Molecular do Paraná Borghetti, Ivo Alberto; Instituto de Biologia Molecular do Paraná; Universidade Federal do Paraná de Souza Luna, Luciano; Instituto de Biologia Molecular do Paraná dos Santos, Alcione; Fundação Oswaldo Cruz Lafontaine, Roger; Centro de Pesquisa em Medicina Tropical, Microbiologia Pereira, Dhelio; Centro de Pesquisa em Medicina Tropical Krieger, Marco Aurelio; Instituto Carlos Chagas; Instituto de Biologia Molecular do Paraná Salcedo, Juan Miguel; Fundação Oswaldo Cruz; Centro de Pesquisa em Medicina Tropical Rampazzo, Rita de Cássia; Instituto de Biologia Molecular do Paraná,
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EPIDEMIOLOGICAL PROFILE OF ZIKA, DENGUE, AND CHIKUNGUNYA VIRUS INFECTIONS IDENTIFIED BY MEDICAL AND MOLECULAR EVALUATIONS IN THE WESTERN BRAZILIAN AMAZON

Deusilene Souza Vieira[2,3,5], Débora da Silva Lopes[2], Lucas Sapiecinski de Oliveira[2], Miriam Ribas Zambenedetti[1], Luciana Requião[1], Ivo Alberto Borghetti[1,4], Luciano Luna[1], Alcione de Oliveira dos Santos[2,3,5], Roger Lafontaine Mesquita Taborda[5], Dhelio Batista Pereira[5], Marco Aurélio Krieger[1,6], Juan Miguel Villalobos Salcedo[2,5], Rita de Cássia Pontello Rampazzo[1]

[1] Molecular Biology Institute of Paraná, Curitiba, Paraná, Brazil. [2] Oswaldo Cruz Foundation of Rondônia, Porto Velho, Rondônia, Brazil.[3]Program of Graduate in Experimental Biology – Federal University of Rondônia, Porto Velho, Rondônia, Brazil. [4]Department of Bioprocess Engineering and Biotechnology, Federal University of Paraná, Curitiba, Paraná, Brazil. [5]Research Center on Tropical Medicine, Porto Velho, Rondônia, Brazil. [6]Carlos Chagas Institute, Curitiba, Paraná, Brazil.

ABSTRACT

We analyzed arboviruses in serum specimens from 164 symptomatic patients in the western Brazilian Amazon, where the epidemiological profile of arbovirus infections is not well defined. Zika virus RNA was detected in 4.3% of the patients, and no study patient was positive for dengue or chikungunya viruses. To our knowledge, this is the first identification of Zika virus infections in the region. Our results indicate that the medical and molecular parameters obtained by our investigations were important for clarifying the epidemiology of arbovirus infections. Furthermore, the low rate of definitive diagnoses suggest that new molecular tests for identifying diseases that manifest similar signs and symptoms should be used for this type of study.

Keywords: Zika, chikungunya, dengue, one-step real-time RT-PCR, diagnostic

1. INTRODUCTION

Climatic conditions together with the large populations of *Aedes* sp mosquitoes in the western Brazilian Amazon lead to the perfect environment for maintaining arbovirus transmission cycles. Outbreaks of dengue, yellow fever, Oropouche, and Mayaro have already been reported from this region[1–3].

The Zika virus (ZIKV) was first isolated in 1947 in Uganda from a monkey, and sporadic infections were subsequently identified in humans. In 2007, the virus was found to be widespread in patients presenting with flu-like signs and symptoms during an outbreak of disease on Yap Island of the Federated States of Micronesia. The subsequent increased incidence of patients with microcephaly or Guillain-Barré syndrome in Brazil was intensively investigated, and the resulting data established an association between Zika virus infections and neurological disorders[4–6].

Dengue fever is the most important re-emerging diseases that is transmitted by the bite of arthropods. Infection with the dengue virus (DENV) normally leads to fever and flu-like signs and symptoms, but also can be fatal in some cases[7,8]. Based on epidemiological investigations reported to the World Health Organization, an estimated 50-100 million cases occur annually in more than 100 endemic countries, which indicates that half of the world's population is at risk[8].

Chikungunya virus (CHIKV) infection was first reported in 1952 in southern Tanzania. Chikungunya disease is characterized by joint pains and high fever. The virus recently arrived in Brazil (2014), and from the first report to the second half of 2017 approximately 300,000 cases had been reported, and 40% of Brazilian municipalities had confirmed cases[9,10].

The aim of this study was to investigate if the main arboviruses spreading in Brazil (ZIKV, DENV, and CHIKV) were present in patients with symptoms of arboviral disease, who were living in the Western Brazilian Amazon (states of Acre, Amazonas, Roraima and Rondônia), during the first half of 2017. In summary, to our knowledge, our study has provided the first epidemiological data on patients in the region, from whom isolates of ZIKV were identified. We suggest that other diagnostic protocols for the diagnosis of arbovirus infections should be combined in order to develop an improved algorithm for identifying specific pathogens.

2. MATERIALS AND METHODS

2.1 Samples: The study was performed in Porto Velho, Rondônia, Brazil, during the first half of 2017, and approved by the local Research Ethics Committee (1.474.102CEP/2016/CEPEM). Serum samples from 164 patients presenting with clinical symptoms of arboviral infection were collected according to outpatient medical procedures performed at the Research Center for Tropical Medicine (CEPEM)/Rondônia, Brazil.

2.2 RNA extraction: Viral RNA was isolated from 140 µL of each serum sample by the QIAamp® Viral RNA Mini Kit (QIAGEN, Germany), according to the manufacturer's instructions. RNA was eluted in 60 µL of AVE buffer.

2.3 Real-time RT-PCR: The RNA samples were tested for the presence of ZIKV; DENV serotypes 1, 2, 3, and 4; and CHIKV by a one-step real-time Biomol ZDC PCR kit (Instituto de Biologia Molecular do Paraná, Brazil), following the manufacturer's instructions. The reactions were performed in an Applied Biosystems 7500 Real-Time PCR System, with the reaction profile: 51°C for 30 minutes, 95°C for 15 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Reactions were considered positive when the cycle threshold was ≤ 35 for the virus analyzed and ≤ 28 for the internal control.

2.4 DENV NS1 antigen testing: Serum samples were tested using the Platelia Dengue NS1 AG BIO-RAD serological test (BIO-RAD, USA), an enzyme immunoassay for qualitative or semiquantitative detection of the NS1 antigen in human serum or plasma.

2.5 Microscopy for malaria: Thick blood smears were stained with Giemsa, the gold standard method. Each blood smear was examined for the presence of malaria parasites by experienced technicians.

3. RESULTS

RNA samples from the sera of 164 patients were tested in a real-time RT-PCR assay. Analyses of the amplification results revealed that 7 (4.3%) samples were positive only for ZIKV RNA, and 157 were negative for all DENV serotypes, CHIKV, and ZIKV RNA. The negative real-time RT-PCR results were validated by the internal control. All RNA samples showed amplification of the internal control sequence (an endogenous human transcript), to ensure that these samples were not degraded and that the negative results were true negatives. The serological test for NS1 DENV antigen was also negative for all serum samples. Microscopic evaluation of 164 peripheral blood smears was also negative for malaria infection.

During anamnesis, the main signs and symptoms reported by our patients that were considered to be relevant to our study were as follows: conjunctivitis 7/164 (4.3%), fever 84/164 (51.2%), headache 78/164 (78%), chills 10/164 (6.1%), pruritus 20/164 (12.2%), exanthema 33/164 (20.1%), arthralgia 58/164 (35.3%), myalgia 44/164 (26.8%), and retro-orbital pain 32/164 (19.5%). Correlations between the signs and symptoms of the patients who were ZIKV positive or negative are shown in Table 1.

Table 1. Signs and symptoms reported by patients

Of the signs and symptoms reported by the patients with viremia, only conjunctivitis had statistical relevance (OR=12.16, 95% CI, 1.88-78.63, p=0.03), with a positive correlation between conjunctivitis and positivity for ZIKV. Therefore, patients with ZIKV infections are more likely to develop inflammatory conditions in the ocular region ($p < 0.05$) than uninfected individuals.

In addition, the specificity of the real-time RT-PCR assay (Biomol ZDC kit) for ZIKV, DENV, and CHIKV was assessed by testing the assay with samples known to be positive for *Plasmodium falciparum*; Mayaro, yellow, and Oropouche fever viruses; and ZIKV, DENV1, DENV2, DENV3, DENV4, and CHIKV. The data showed specific amplifications only, confirming the absence of assay cross-reactivity with the evaluated pathogens. The assays were validated by the positive control supplied by the manufacturer.

4. DISCUSSION

Infections with arboviruses and malaria occur in the same geographical regions because of the association between the climate and presence of the vectors of the pathogens, *Aedes* and *Anopheles* sp, respectively. Patients with these infections present with similar signs and symptoms, including chills, fever and headache, which may be mild and difficult to recognize[3,11].

We used medical, molecular, and microscopic diagnostic evaluations of 164 patients with signs and symptoms suggestive of arbovirus infections in our study and were able to identify the first cases of Zika virus infections in the western Brazilian Amazon by a one-step real time RT-PCR assay, after a worldwide explosive outbreak of the virus in 2015. The 4.3% detection rate of viral ZIKV RNA in patients in this region was surprising, because until our positive findings, there had not been any confirmed cases in this region. In addition, by the first half of 2017, the virus seemed be controlled in Brazil. The nucleic acid assay that we used to detect ZIKV was used effectively in another earlier epidemiological study to detect ZIKV RNA in different types of specimens, which included urine and saliva[12].

Several previous screening studies had been carried out for ZIKV, DENV, and CHIKV infections in some localities in northern Brazil; however, no screening study for ZIKV had been performed in the western Brazilian Amazon. Currently, there are ongoing surveys of DENV and more recently for CHIKV infection (e.g., first description of CHIKV in Amapá state)[13].

The duration of time from the first molecular detection of ZIKV in the western Brazilian Amazon to the first reported detection of autochthonous ZIKV in Brazil[14] suggests that this virus is being established in this region, as has been already described for DENV and CHIKV. Our new findings should be of paramount importance to the public health authorities, since infection with ZIKV, as observed mainly in the Northeast region, has been responsible for the occurrence of complicated neurological syndromes in Brazil[15].

In this study, the symptoms considered relevant for arboviral infection, which were correlated with all the cases confirmed positive for ZIKV RNA by molecular assay, should be presumptive for ZIKV infection. Because of the dispersion of specific vectors for ZIKV over the entire country, molecular screening should be considered an important tool for monitoring patients with acute infections. Finally, the low rate of detection of ZIKV-infected individuals (7/164) and the absence of cases positive for DENV, CHIKV, and malaria infections suggest

that other pathogens have been circulating in this area. Because many outbreaks of arbovirus infections in the western Brazilian Amazon have been reported in the past, medical and molecular investigations for new arboviruses that are spreading across this region are needed. These viruses include the West Nile, Mayaro, yellow fever, and Oropouche.

5. CONCLUSION

To the best of our knowledge, this study has produced the first molecular evidence of human Zika virus infections in the western Brazilian Amazon, a region considered highly endemic for arboviruses. Since the signs and symptoms presented by our study population are manifestations of various arboviruses, an accurate laboratory diagnosis is needed. We hope that our data will contribute to the facilitation of clinical and epidemiological decision making.

6. CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflicts of interest.

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5 CONSIDERAÇÕES FINAIS

O controle positivo construído atendeu o objetivo do trabalho pois, quando testado em reações de RT - qPCR em tempo real para o qual foi estabelecido, apresentou amplificação específica para os alvos avaliados.

Este produto (kit Biomol ZDC com o controle), desenvolvido com insumos e tecnologia nacionais apresenta um custo menor e possui enorme valor agregado, estando em fase de registro, e com potencial para atender as necessidades da Saúde Pública brasileira.

5.1 RECOMENDAÇÕES PARA TRABALHOS FUTUROS

A construção desse controle contendo 7 alvos demonstrou a importância, inclusive frente a literatura que normalmente usa 1 alvo.

Desta forma, as recomendações para ampliações futuras para uso de controles semelhantes encontram-se nos estudos de RT- qPCR multiplex que o Instituto de Biologia Molecular do Paraná já está desenvolvendo.

Assim sendo recomendamos que este modelo de controle externo positivo pode ser utilizado para outras metodologias desenvolvidas e aplicado em outros microrganismos de interesse para a saúde pública.

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ANEXOS

Physiol Mol Biol Plants
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RESEARCH ARTICLE

Efficient genetic transformation and regeneration system from hairy root of *Origanum vulgare*

Peyman Habibi[1,2] · Maria Fatima Grossi de Sa[1,2,3] · André Luís Lopes da Silva[1] · Abdullah Makhzoum[4] · Jefferson da Luz Costa[1] · Ivo Albertto Borghetti[1] · Carlos Ricardo Soccol[2]

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Abstract *Origanum vulgare L* is commonly known as a wild marjoram and winter sweet which has been used in the traditional medicine due to its therapeutic effects as stimulant, anticancer, antioxidant, antibacterial, anti-inflammatory and many other diseases. A reliable gene transfer system via *Agrobacterium rhizogenes* and plant regeneration via hairy roots was established in *O. vulgare* for the first time. The frequency of induced hairy roots was different by modification of the co-cultivation medium elements after infection by *Agrobacterium rhizogenes* strains K599 and ATCC15834. High transformation frequency (91.3 %) was achieved by co-cultivation of explants with *A. rhizogenes* on modified (MS) medium. The frequency of calli induction with an 81.5 % was achieved from hairy roots on MS medium with 0.25 mg/L^{-1} 2,4-D. For shoot induction, initiated calli was transferred into a medium containing various concentrations of BA (0.1, 0.25, 0.5, 0.75 and 1 mg/L^{-1}). The frequency of shoot generation (85.18 %) was achieved in medium fortified with 0.25 mg/L^{-1} of BA. Shoots were placed on MS medium with 0.25 mg/l IBA for root induction. Roots appeared and induction rate was achieved after 15 days.

✉ Abdullah Makhzoum
abmakhzoum@gmail.com

✉ Carlos Ricardo Soccol
soccol@ufpr.br; carlosricardo.soccol@gmail.com

[1] Bioprocess Engineering and Biotechnology Department, Federal University of Paraná, Curitiba, Paraná, Brazil

[2] Embrapa Genetic Resources and Biotechnology, Brasilia, DF, Brazil

[3] Catholic University of Brasília, Brasília, DF, Brazil

[4] Department of Biology and the Biotron, The University of Western Ontario, London, ON N6A 5B7, Canada

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Abbreviations

2,4-D	2,4 dichlorophenoxyacetic acid
BA	6- Benzyladenine
NAA	α-Naphthalene Acetic Acid
IBA	Indol-3- Butyric Acid

Introduction

Origanum vulgare L. belongs to the family of Lamiaceae and well-known for its essential oils, including phenolic monoterpenoids which are used as food flavors. This species is commonly known as a wild marjoram/winter sweet and has been utilized in medicine due to its anti-inflammatory, anticancer and antibacterial effects (Béjaoui et al. 2013; de Souza et al. 2016; Govindarajan et al. 2016; Pahlavan et al. 2013). Therefore, large-scale production of secondary metabolites of this plant using cell culture might open up a new avenue for the commercialization of its valuable metabolites for pharmaceutical industry. Recently, various methods have been employed to control plant sources for secondary metabolites production at commercial scale, including callus cultures, cell suspension culture, root cultures, organogenesis and embryogenesis (Paul et al. 2011). Hairy root system is one of the interesting aspects of plant cell cultures which provide an effective understanding of the physical and chemical requirements, growth and development of plant cells. Basically, hairy roots are induced through the integration of *rol* genes of *Agrobacterium rhizogenes* in the chromosome region of plant cell cultures. (Georgiev et al. 2010). When various susceptible parts of plants, such as the leaf, shoot and root, are wounded,

they excrete simple phenolic substances, such as acetosyringone which subsequently promote induction of virulence (vir) genes which are involved in the excision and transfer of the T-DNA fragment. This process results in generation of hairy roots at the wounded site (Makhzoum et al. 2013). Additionally, this platform has been investigated in plant secondary metabolism (Harfi et al. 2015; Makhzoum et al. 2015, 2011) as well plant molecular pharming and the production of heterologous recombinant pharmaceutical proteins and enzymes (Makhzoum et al. 2014; Moustafa et al. 2015) and phytoremediation. Since the hairy root platform presenting added advantages, such as, genetic stability, fast growing specially on hormone free medium, it can help in the development of affordable cost production by increasing of yield and ease of management, they could be considered as an attractive system to substitute current plant cell culture systems as the last ones are criticized for their genetic instability and low production rate (Georgiev et al. 2012). Hairy roots induction by *Agrobacterium*-mediated transformation opens up new windows for various fundamental applications in plant genetic engineering. This interesting system could allow modifications and production of phytomolecules that cannot realistically produce via chemical semi-synthesis. Interestingly, hairy root platform can offer a reliable protocol for regeneration in plant transformations as improvement in in vitro regeneration systems and feasible stable genetic transformation systems represent the main obstacle for successful plant genetic modifications (Al-Shalabia et al. 2014; Chandra and Chandra 2011; Yang et al. 2013; Zhang et al. 2007). In this context regeneration of whole plant via hairy roots has already been achieved in some species including: *Ipomoea batatas* and *Ipomoea trichocarpa* (Otani et al. 1993, 1996), *Brassica campestris* and *B. oleracea* (Christey et al. 1997), *Crotalaria juncea* (Ohara et al. 2000), *Panax ginseng* (Yang and Choi 2000) etc. Considering the importance of the subject, we highlight a reliable system for *A. rhizogenes*-mediated *O. vulgare* transformation to investigate the effect of medium composition modifications on hairy root induction frequency. Here we report for the first time the hairy root induction and the regeneration of the medicinal plant *O. vulgare* by *A. rhizogenes* as well as optimization of medium composition.

Material and methods

Plant materials and tissue culture

Seeds of *O. vulgare* were washed with running tap water for 20 min and were surface sterilized with 70 % ethanol for 1 min and subsequently suspended on 2 % sodium hypochlorite for 12 min. Thereafter, seeds were washed 3–4 times with sterile distilled water. Half strength MS (Murashige and Skoog 1962) medium without plant growth regulator, containing 30 gL^{-1} of sucrose was used for seed germination. Before seed culture, the medium was adjusted to pH 5.7 and autoclaved under 1.06 kg/cm^2 pressure at 121 °C for 15 min. Cultures were incubated and maintained at 26 ± 1 °C and for 16 h photoperiod.

A. rhizogenes preparation

Strains of *ATCC15834* and *K599* were inoculated in YEB agar medium (Vervliet et al. 1975) containing 5 g/L beef extract, 0.5 g/L MgCl2, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 15 $g.L^{-1}$ agar and pH 7.2 to an optical density of 0.6, at 28 °C, 120 rpm on a shaker incubator for 24 h. The strains were pelleted by centrifugation for 15 min at 3000 rpm and resuspended to a cell density of OD_{600} = 0.8 in a co-cultivation medium that consisted of MS with salts and vitamins, supplemented with 50 mg/L sucrose.

Induction of hairy roots and culture conditions

In order to induce hairy roots, leaves were isolated from 20 to 25 day old plants and were cut into 2–3 cm segments and randomly wounded by using a sterile needle and immersed in the *Agrobacterium* suspensions and swirled in liquid inoculation medium for10 min. The explants were blotted to dry on sterile filter paper and then transferred to a co-cultivation medium in the dark. In this experiment, the medium used for co-cultivation contained MS salts, vitamins, 30 gL^{-1} sucrose, 100 µM acetosyringone and 7 gL^{-1} agar (designated as Medium 1). This medium was finally modified by removing the following salts: KH_2PO_4 (co-cultivation medium 2); KH_2PO_4, $CaCl_2$ (co-cultivation medium 3); KH_2PO_4, NH_4NO_3, KNO_3 (co-cultivation medium 4); KH_2PO_4, NH_4NO_3, KNO_3, $CaCl_2$ (co-cultivation medium 5); KNO_3, $CaCl_2$, KH_2PO_4, Na_2EDTA, NH_4NO_3, $MgSO_4$, $FeSO_4$, vitamins and microelements (co-cultivation medium 6). Approximately, 40 to 50 leaf explants were inoculated with *A. rhizogenes* for each treatment in each experiment in dark conditions for 2 days at 28 C. After 2 days of co-cultivation, the explants transferred to MS media supplemented with 300 mgL^{-1} cefotaxime® to remove the *Agrobacterium* for 14 days. Hairy roots of *O. vulgare* were produced after 2 weeks and cultivated in hormone-free medium, containing 30 gL^{-1} of sucrose supplemented with 200 mgL^{-1} cefotaxime to establish axenic transformed root cultures for 15 days. The amount of cefotaxime in the culture medium was gradually decreased to 50 mgL^{-1} after 60 days. Induced hairy roots were subcultured every week. The selection of induced hairy roots was performed based on fast growth and shape and then they brought onto a 100 mL MS liquid medium in 250 ml flask and

kept at 110 rpm for further research. These conditions were kept for control explants but except inoculation with A. *rhizogenes*.

Callus induction from hairy roots

Induced hairy roots (5 g) were cut into segments and cultured on solid callus induction media. The callus induction medium contained MS micro and macro- nutrients which was supplemented with different levels (0.0, 0.1, 0.5, 0.75 and 1.0 mg/L) of 2,4-D.

Shoot regeneration from callus derived from hairy roots

The induced callus in the previous step was excised into 2–3 cm segments and transferred onto MS solid medium supplemented with 3 % sucrose, 0.1, 0.25, 0.5, 0.75, and 1 mg/L BA and pH was adjusted to 5.8. After 3 weeks, the regenerated shoots (2–2.5 cm) were excised and transferred to a root induction medium (MS medium supplemented with 0.5 mg/L IBA).

Polymerase chain reaction analysis

Extraction of genomic DNA from hairy and normal roots (negative control) of *O. vulgare* was performed by DNA extraction kit (Qiagen, Germany) based on manufacturer's instructions. The plasmid DNA of A. *rhizogenes* strain was also extracted with the plasmid isolation kit (QIAGEN, Germany) according to manufacture instruction. The DNA samples were run to PCR amplification with specific primers for the confirmation of the *rolB* gene (forward primer 5'ATGGATCC CAAATTGCTATTCCCCCACGA-3' and reverse primer 5'TTAGGCTTCTTTCATTCGGTTTACTGCAGC-3') according to (Ahmadi Moghadam et al. 2013) under the following conditions: 94° for 7 min, followed by hot start at 94 °C for 10 min and amplified during 30 cycles at 94 °C for 45 s, 52 °C for 55 s, 72 °C for 1 min and followed by a final extension step at 72 °C for 5 min. The electrophoresis of the PCR products was performed on 1.2 % agarose gel under a constant voltage of 80 V.

Southern hybridization

Rapid genomic DNA extraction of transgenic hairy roots and non-transgenic roots of *O. vulgare* was carried out by DNA extraction kit based on manufacturer's instructions and then 20 µg of extracted DNA was digested by XbaI (Fermentas Co., Germany) and the separation of digested DNA was performed by 0.8 % agarose gel and finally shifted to a nylon membrane. Probe was prepared based on *rolB* sequence, using the DIG DNA labeling Kit (Roche Co., Germany). Prehybridization, hybridization, washing and detection were performed based on the DIG Labeling and Detection System instruction manual of (Roche Co., Germany).

Statistical analysis

The experimental design used in our experiments was completely randomized. So, each treatment was consisted of six replications and nine explants that were cultured in each Petri dish. The data was submitted to the analysis of variance (ANOVA) and to normality analysis for the Lilliefors test. Regression analysis was used to study Quantitative data while qualitative data were analyzed by the Scott-Knott test. All statistical analysis were performed at a level of $P < 0.05$. The SOC software was employed to perform all statistical analyses at a level of $P < 0.05$ and variables from percentages were transformed to arcsin x /100.

Results and discussion

The effect of bacterial strains and media compositions

Agrobacterium rhizogenes strains: 15834 and K599 were investigated to check their ability for transformation. Both strains were capable to induce hairy roots 2 weeks after inoculation directly from wounded sites (Fig. 1a). Wounded shoot explants were significantly receptive to transformation by *A. rhizogenes*. The highest rate of infection (91.3 %) was gained by two strains in leaves explants during 4 weeks on medium 5 whereas medium1 has shown the lowest rate of hairy root induction (34.6 %) via two strains (Fig. 2).

Callus induction from hairy roots

We attempted to induce callus from hairy roots explants by using different concentrations of 2–4-D (Fig. 1b). After 2 weeks, the highest frequency (81.5 %) of callus induction was achieved from hairy roots on MS medium containing 0.25 mg/L 2,4-D, and the lowest induction frequency (38.3 %) was achieved on a medium containing 1 mg/L 2,4-D as shown in (Fig. 3).

Shoot regeneration from callus raised from hairy roots

In this present, we could not obtain shoot induction directly from hairy roots without an intervening callus phase (data not shown). In order to establish shoot regeneration, segments of the induced callus from a hairy root clone were transferred to MS medium in different concentrations of BA. Shoot formation of *O. vulgare* was initiated from callus (Fig. 1c). The frequency of shoot induction in media with different concentrations of BA was different after 3 weeks of cultivation. The maximum frequency (85.18 %) of shoot formation was

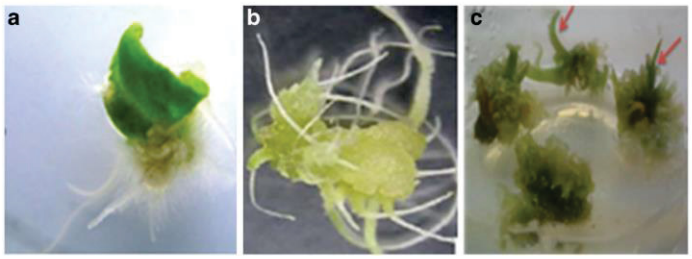

Fig. 1 **a** Hairy root induction of *O. vulgare*, **b** callus induction from a hairy root of *O. vulgare* **c** induction of an adventitious shoot from a hairy root of *O. vulgare*

achieved in a medium with 0.25 mg/l BA and the lowest frequency (24.69 %) with a concentration of 1 mg/L BA was detected (Fig. 4).

Molecular analysis of transgenic hairy roots

Selected hairy root samples were subjected to PCR analysis. PCR results showed the presence of the *rolB* gene in transformed clones (Fig. 5). The integration of the *rolB* gene was further confirmed by Southern-blot analysis and also confirmed the insertion of T-DNA into hairy root genome, since non transformed roots showed the integration signal (Fig. 6).

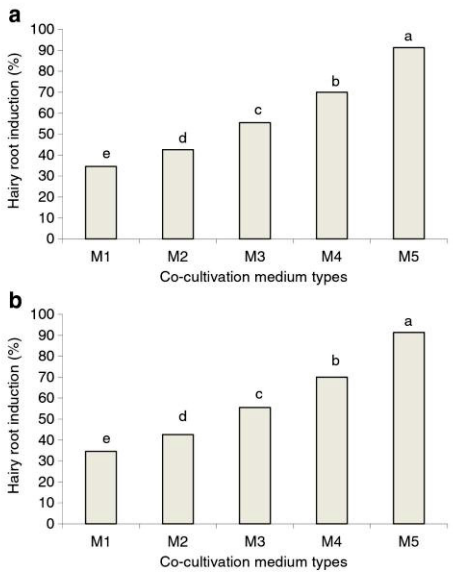

Fig. 2 The effect of different co-cultivation media on percentage of hairy root induction in *O. vulgare* by strain of ATCC15834 (**a**) and the effect of different co-cultivation media on percentage of hairy root induction in *O. vulgare* by strain of K599 (**b**)

These results represent a sustainable conjugation of TL-DNA of *A. rhizogenes* into hairy root chromosomal genome (Jouanin et al. 1987; White et al. 1985).

The efficiency of the genetic transformation of a plant species via *Agrobacterium* is associated with the type of the culture medium, temperature, co-culture time, genotype, and *Agrobacterium* strain (Lopes da Silva et al. 2013). Previously, the capability of various strains of *A. rhizogenes* has been reported to influence induction and development of hairy roots. *A. rhizogenes* strains ATCC15834 and K599 belong to the agropine-type Ri-plasmids, which are very similar as a group (Sevón et al. 2002). Trypsteen et al. (1991) showed that the overall process of T-DNA integration in an agropine type is very similar (Trypsteen et al. 1991). Tinland (1996) showed that the age and differentiation status of plant tissue and the level of tissue differentiation can affect hairy root induction after inoculation with *A. rhizogenes* (Tinland 1996). Porter (1991) believes that the capability of *A. rhizogenes* to transform plant species rely on the strain, but Lee et al. (2010) concluded that the effectiveness of *Agrobacterium* strain to induce hairy roots potentially rely on plant species (Lee et al. 2010; Porter 1991). Removing KH_2PO_4 and $CaCl_2$ or NH_4NO_3, KH_2PO_4 and KNO_3 in medium 3 or 4, respectively, obviously promoted root induction frequency. It has been recommended that the low concentration of PO_4 may influence the expression of virG and release positive signal to facilitate infection process (Sharafi et al.

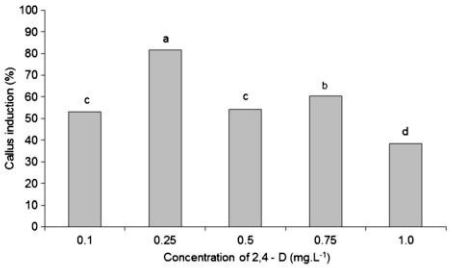

Fig. 3 Concentration of 2, 4-D in an MS medium on a frequency of adventitious callus induction in *O. vulgare*

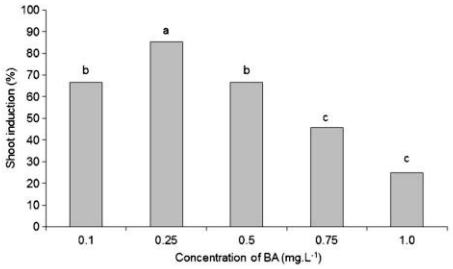

Fig. 4 Effect of different concentrations of BA in an MS medium supplemented with 0.1 mg/l NAA on frequency of adventitious shoot induction in *O. vulgare*

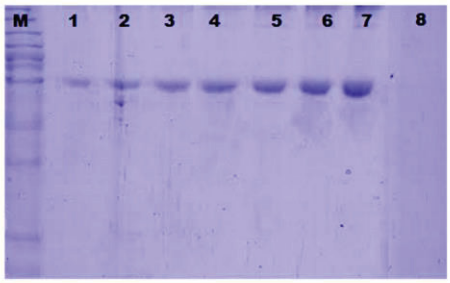

Fig. 6 Confirmation of transgenic nature of hairy roots by southern hybridization. Lanes 1–6 Genomic DNA isolated from transgenic *O.vulgare.* Lane *7 Agrobacterium rhizogenes* plasmid as a positive control, lane 8 Non-transformed plant genomic DNA as negative control

2012). Also, in comparison to the full-strength MS medium (Medium 1), in Medium 5, hairy root inductions were significantly increased by removing $CaCl_2$. Medium with high salt concentration such as, MS may divest the process of root development due to lacking of sufficient water and nutrients absorption from medium. That while a lacking mineral components medium was used for co-cultivation, a high transformation rate of *Ginkgo biloba* was achieved (Dupre et al. 2003). Thus, the concentration of components involved in inoculation and co-cultivation media may affect the frequency of transformation of *O. vulgare* explants by *A. rhizogenes.* The transformation efficiency significantly increased through a change in some major mineral components. Bacterial multiplication and hairy root formation would be influenced in a low-salt medium (Azadi et al. 2010). Key factors as the capacity of a calcium inhibitory effect and lacking PO_4 in activating virulence genes as well as the possibility of biofilm formation have also been demonstratedto influence *Agrobacterium*-mediated transformation (Danhorn et al. 2004; Flego et al. 1997; Winans 1990). However, more investigations on the effect of these components are needed to

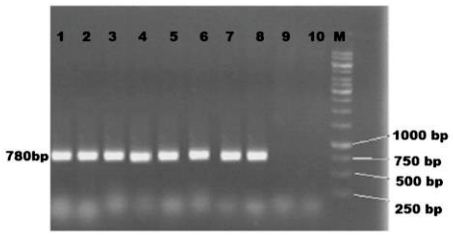

Fig. 5 PCR analysis for detection of the *rolB* gene in hairy roots lines and regenerated callus and shoot of *O.vulgare*. Lane 1:*Agrobacterium rhizogenes* DNA (positive control); Lanes 2-6 hairy root lines; Lane 7 regenerated callus from hairy root of *O.vulgare;* lane 8 regenerated shoot from hairy root of *O.vulgare;* lane 8,9 = none transformed explant (negative control); Lane M : Marker

determine how gene transfer is influenced by mineral elements. These results are in agreement with the results obtained by other experiments in other plant species (Azadi et al. 2010; Hoshi et al. 2004; Sharafi et al. 2012). The induced callus via hairy root of *O. vulgar* (Fig. 1b) may be the result of its physiological state, which provides actively dividing cells. The development of a callus from hairy roots is directly related to the presence of 2,4-D, which is one of the favorable growth hormone for callus induction in most plants. The wounding during the process of cutting resulted in asynchronous cell division. This is considered to be a process of de-differentiation of organized tissue. Previously, the assessment of plant growth hormones effect on induced callus from hairy roots in *pumpkin* (*Cucurbita pepo* L.) represented inhibitory effect of low2,4-D concentration on root proliferation while exposed positive effect on influence of callus induction (Katavic and Jelaska 1991). In another study on *crownvetch* (*Coronilla varia* L.) carried out by Han and his co-workers, hairy root induction and plant regeneration was achieved by using *A. rhizogenes*. The results of the study showed that the callus induction with 100 % frequency was achieved from hairy roots in an MS medium containing 0.2 mg/L 2,4-D (Han et al. 2006). In another work done by Wang and his collaborators, the maximum frequency of callus induction from hairy root has been established in medium with 2.0 mg/L 2, 4-D and 0.5 mg/L 6-BA. The callus establishment was gained after 28 days, and transferred to an MS medium to facilitate shoot regeneration (Wang et al. 2001). Kumari and Saradhi (1992) reported that the best callus induction of *Origanum vulgare* was noted in a medium with 2, 4-D alone (Kumari and Saradhi 1992). Embryogenic studies of *Beta vulgaris L.* by callus induced from hairy roots system has been reported by Ninković et al. (2010). They concluded that the maximum callus frequency could be retrieved on MS medium supplemented with 1 mg/L thidiazuron (TDZ) and 1 mg/L 2, 4- D (Ninković et al. 2010).

It was shown that the best shoot induction of *O. vulgare* was obtained in a medium supplemented with BAP and NAA (Kumari and Saradhi 1992). The concentration of 0.5 mg/L BA was recommended to obtain maximum shoot regeneration from callus of *Populus tremuloides Michx* (Noh and Minocha 1986). In this case, our studies are similar and in agreement with (Han et al. 2004). They also remarked BA as an effective hormone involved in shoot regeneration. Similar results have been drawn in another study on the *bottle gourd* that explains BA is essential for shoot bud formation (Saha et al. 2007). In our experiments, shoot formation was not detected on the hormone-free medium, which is in consistence with the results obtained by *Pogostemon cablin* and *Dracocephalum kotschyi* (He-Ping et al. 2011; Sharafi et al. 2014), respectively. In summary, the insertion of native *A. rhizogenes* T-DNA into the *O. vulgare* genome worked successfully. Our data shows that regeneration is possible from hairy roots, but there is no significant difference between strains of bacteria K599 and ATCC15834 on hairy root induction of this plant. The co-cultivation of bacteria with explants in MS medium lacking macro elements is the best condition for the induction of hairy roots in *O. vulgar*. Thus, it could be concluded that macro elements significantly affect hairy root induction. Since transgenic shoots of *O. vulgare* on PGR-free medium are impoverished to generate root (Fig. 5c), they were placed on MS medium supplemented with 0.25 mg/L IBA. Root induction (90 %) appeared from the bottom of the shoots after 2 weeks. Finally, for the first time, we report hairy root induction and plant regeneration from induced hairy roots of *O. vulgare* and the results of these experiments could be used to study and scale up valuable secondary metabolites by this plant in the future. Moreover, the possibility of regenerating *O. vulgare* plants from hairy roots may allow for a higher production of secondary metabolites through genetic engineering. Therefore, the study of valuable secondary metabolite production of *O. vulgare* using hairy roots may lead to a higher production of these bioactive compounds.

Conclusion

In conclusion, the native A. *rhizogenes* T-DNA has been successfully inserted into the *O. vulgare* genome in active positioning state. In this research, we have established a well-founded protocol to induce transgenic hairy roots from *O. vulgare* and then plant regeneration from induced hairy roots was obtained by different concentrations of plant hormones. Results of this work could potentially improve the production of valuable secondary metabolites in this plant as large scale production of these secondary metabolites has been a priority in plant cell culture system and phytochemical industry.

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Review

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Biorefinery Integration of Microalgae Production into Cassava Processing Industry: Potential and Perspectives

Júlio Cesar de Carvalho*, Ivo Alberto Borghetti, Liliana Carrilo Cartas, Adenise Lorenci Woiciechowski, Vanete Thomaz Soccol, Carlos Ricardo Soccol

Bioprocess Engineering and Biotechnology Department, Federal University of Paraná (UFPR), Centro Politécnico, C.P. 19011, 81531-980 Curitiba, PR, Brazil

*corresponding author at jccarvalho@ufpr.br

Abstract

Cassava, the 5th most important staple crop, generates at least 600L of wastewater per ton of processed root. This residue, cassava processing wastewater (CPW) has a high chemical oxygen demand, that can reach 56g/L, and has also high concentrations of several mineral nutrients. The cultivation of microalgae such as *Chlorella*, *Spirulina* and wild strains was evaluated in the last years in raw, minimally processed and partially digested CPW. Concentrations of 2 to 4 g/L of these microalgae, comparable to those obtained in synthetic media, could be reached. The BOD of the residue was reduced up to 92%. This process can be integrated into cassava processing industries, if challenges such as the toxicity of the concentrated residue, bacterial contamination, and the isolation of robust strains are addressed. Because CPW carries about 11% of the crop energy, integrating biogas production and microalgal cultivation into the cassava processing chain is promising.

Key words: microalgae, cassava, wastewater, manipueira, biodigestion

1. Introduction

Cassava is the 5th most important staple crop in the world, with a production about 285 million tons/year of unprocessed roots (FAOSTAT, 2017). Cassava industrialization generates large amounts of byproducts. The solid residues can be used as fodder, used as solid substrates for fermentation, or biodigested for energy production. The liquid residues, however, are the larger part of the residue and have a high pollution potential.

Cassava processing wastewater (CPW, also called *manipueira* in Brazil), is the mixture of process water (for root and starch washing) with the water liberated from disintegration of cassava. Several substances are carried with the residue, such as carbohydrates, nitrate, proteins, phosphate, potassium and cyanide. CPW is highly polluting and must be treated before directing it to a water body. The usual treatment is a system of anaerobic, facultative and aerobic ponds, and a final polishing step. However, CPW can be processed into several products such as biogas, ethanol, other fermentation products and microalgal biomass.

Microalgae, which are photosynthetic microorganisms capable of mixotrophic growth, may be adapted to grow in raw or pretreated CPW, and the resulting biomass may be used as food and feed supplements and as raw material for production of concentrated proteins, lipids and carbohydrates. This work reviews some basic aspects about cassava production and processing, the residues generated, introduces the concept of microalgal cultivation and its isolation from environmental samples, and shows some results obtained in the last years by our group, evaluating the culture of *Chlorella minutissima*, *Spirulina platensis*, *Neochloris oleoabundans* and native

microalgae in cassava processing wastewater. A final outlook of the future integration and challenges is presented.

2. Cassava processing and residues

Cassava is a staple crop produced in tropical areas around the world. Rich in starch, the plant is an important source of energy in diets. Despite being native to South America, Nigeria is the largest cassava producer, followed by Brazil, Thailand and Indonesia. With a global planted area around 2.5 million ha and an average productivity of 11.4 t/ha, cassava is a good nutritional source of carbohydrates and a fair source of proteins. Actually, cassava leaves, that are edible and represent 44% of the whole plant mass, have a much higher content of proteins (23%, versus 1.75% in the root, in dry basis) (De Carvalho et al., 2011), and may increase in importance as a processed food. The crop has a relatively low potassium and nitrogen fertilizer requirement, but the addition of phosphates enhances the productivity (Silva et al., 2013). When produced after another crop such as corn, the residual fertilizer in the soil is often enough for cassava cultivation. The vegetative cycle depends on the variety, climate and destination, but is typically around 1 year for *in natura* use and 2 years for industrial use.

Part of the crop is marketed *in natura*, and is used in several dishes. Most of the crop is processed, mainly into flour and starch, and to a lesser extent into cassava chips and traditional foods. These processes require peeling, grating and pressing or washing steps, in some cases after fermentation. Table 1 shows the main processing steps for the most important cassava products.

Table 1. Main steps for cassava processing. The steps (gray where applicable) should be read from top to bottom. Disintegration of the pressed mass and classification after drying is usual.

Step	Flour (Worldwide)	Water flour Brazil / Oyek Indonesia/ / Fufu Nigeria	Starch (Worldwide)	Sour starch (Brazil)	Gari (Africa)	Residues
Wash						Wastewater (low BOD)
Peel						Peel
Ferment		4 days				Wastewater
Grate						
Press					With fermentation	Wastewater, (high BOD)
Wash fibers						Wastewater
Concentrate						Wastewater
Ferment				15 days	3 days	
Dry/Toast	110 - 160°C	160°C	60-110 in Flash driers	sun	160°C, often with oil	

Adapted from (De Carvalho et al., 2011)(Awoyale et al., 2017) and (FAO/OMS, 2013). Some parameters such as drying/toasting fermentation depend on the variety within a specific product.

Other products are made from the extracted starch: starch pearls (called sago after the similar product obtained from the sago palm) and tapioca (granulated and hydrated starch, popular in Brazil). Because cassava processing is relatively simple, there are a lot of small producers worldwide; most of the crop is processed in large industries, though, that buy the root from producers and benefit from lower specific operational costs, typical of large production scales.

Highlights

- Cassava processing waste (CPW) is generated annually in a volume of 170 billion liters
- 11% of the crop energy is lost in CPW.
- Biogas production from CPW can be integrated into microalgae culture
- Wild isolates grow in raw and pretreated residue, effectively treating it.

Table captions

Table 1. Main steps for cassava processing. The steps (gray where applicable) should be read from top to bottom. Disintegration of the pressed mass and classification after drying is usual.

Table 2 – Composition of cassava processing wastewater from starch and flour industry

Figure Captions

Figure 1 – Main nutrients in CPW and popular culture media. Values are in mg/L. BG11, AS, Spirulina and Bold 3N recipes are from UTEX (2017).

Figure 2 – Chlorella minutissima growth in different concentrations of CPW diluted with MBM medium

Figure 3 – Material balance and energy content in starch production from cassava (in black), with integration of a biodigestion and microalgal biomass unit (in green). The inputs in red are note necessary in an integrated process.

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and cyanide can be reached. Existing industries can profitably treat CPW by using a biodigester to recover energy, while reducing the turbidity and carbon load, followed by microalgal cultivation.

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demonstrated. The intentional use of consortia may allow that microalgae that require specific cofactors grow properly, expanding the possibilities of value-added biomass and products that can be obtained in the process. To develop mixed culture cultivation, it is essential to understand the interactions between microalgal and beneficial bacterial species. With the popularization of molecular techniques for evaluation of mixed microorganism populations, it is expected that new developments occur in this area.

6.5 Integration to biohydrogen production

Biohydrogen production using CPW leads to a liquid stream rich in organic acids, mainly lactic, acetic, butyric and propionic acids. These substances may be used as a carbon source by a microalgal culture, but depending on the concentration will present toxicity. The integration of microalgal cultivation into biohydrogen production calls for specific strain development.

7. Conclusions

CPW is too polluting and too valuable to be discarded. The growth of microalgae in this residue is an alternative for integration into a cassava biorefinery for food or energy production. Microalgae such as *Chlorella* and *Spirulina,* or isolates from the very ecosystems where the cultivation takes place, grow well on pretreated CPW. Even with raw CPW, a COD reduction of 89-99%, and a similar reduction in phosphate, nitrate

tropical areas, where temperature and irradiance are high, but precipitation is also high in part of the season. The oscillation in temperatures and pluviometric indexes, together with the natural oscillation dynamics of mixed populations, may cause cultures to be contaminated or crash if not properly monitored (microbiological and chemical parameters) and controlled (inoculation, controlling agents).

6.3 Species isolation

The selective pressure of the environment in existing CPW treatment systems probably led to microalgae adapted to the local conditions. This includes resistance to cyanide, use of ammonia (after anaerobic systems) and coexistence with bacteria. Microalgae isolated from these environments may be a better fit for cultivation in CPW or DCPW. It is essential to select, among these adapted microorganisms, those with higher growth rates and that are capable of accumulating substances of interest such as dietary lipids, proteins, or carotenoids. Sampling, enrichment an isolation in CPW are the obvious strategies for new strain development. Also, microalgae isolated from CPW lagoons are probably cyanotolerant. Both the extent of resistance and the fate of cyanide (its eventual assimilation) must be better investigated.

6.4 Cocultures and consortia

If you can't beat them, join them. The same goes with microalgae, and there is a trend in recognizing that cocultures and polycultures actually work in large scale. In the case of CPW processing, the use of microalgae-bacteria consortia has already been

The integration of this stream to biodigesters and microalgae cultures, its pretreatment, the fate of the microalgal biomass and the biodigester sludge, and the use of CO_2 from the biogas or burners must be evaluated using multicriteria analysis, taking into account energy recovery, GHG emissions, and capital expenditures that are adequate for the size and technology of the industry.

The use of cassava and its solid agroindustrial byproducts such as cassava bagasse opens yet new possibilities for coupling microalgae production to cassava industry, using stillage from ethanol production as a co-substrate. There is even less research in microalgae cultivation in cassava stillage, although the residue composition and prior work on other ethanol industry residues point to a feasible process.

6.2 Succession in natural environments

The large dimensions of wastewater-based microalgal cultures require these to be, in general, open photobioreactors that are naturally susceptible to contamination. In these systems, the maintenance of unialgal cultures is difficult, and maintenance of axenic cultures is almost impossible. At the other side, axenic cultures are not required if the contaminants are not harmful, and a paradigm shift in cultivation of mixed or polycultures is gaining momentum: in this decade, the terms *coculture* and *polyculture* already appeared in 64 and 8 Scopus-indexed documents, respectively, in association with *microalgae* – a 900% increase in comparison with the prior decade. The study of the succession of microalgae and other species in stabilization ponds, in the existing CPW systems, may lead to a better understanding of the ecology of the system and to what can be expected in large volume cultures. Cassava is produced and processed in

nutrients that were harvested with the roots are fixed in the microalgae and can be marketed as high value co-product, with a production of at least 50 to 100 kg/ha equivalent of microalgal biomass, considering 2 to 4g/L of biomass concentration. Another possibility is the use of microalgal biomass as co-feed for the biodigester, in the case of co-generation. This has the advantage of a simplified downstream process, and can be a first step in the adaptation of an existing plant to a zero-waste biorefinery.

6. Outlook

There are several aspects of microalgal cultivation that need development for successful integration into cassava processing. Besides the evaluation of adequate arrangements for a specific location such as use of raw or digested CPW, recycle for biogas or separation as co-product, etc., there are process-specific aspects that important, yet poorly studied:

6.1 Integration pathways

The possibilities of integration of microalgal production into cassava processing are diverse. Because current starch processing demands for relatively large amounts of water, CPW will continue to exist as a process stream that has an important share of the carbon, nutrient and cyanide content of the root.

Digested CPW has a low suspended solids content and a high concentration of nitrogen, phosphorus and other nutrients, making it an adequate culture medium for microalgal growth. Cartas (2017) compared the growth of microalgae in raw and digested CPW and found similar yields for 4 native species.

The main effect of biodigestion of CPW is the gasification of organic carbon, and therefore nutrient conservation is to be expected. Part of the organic and nitrate nitrogen may be converted into ammonia and volatilized depending on the pH, but the digested CPW still has a N:P ratio around 150 (calculated based on data from (Xie et al., 2014)(Ribas et al., 2010)), still favorable for microalgal development and still phosphate-rich. In fact, the accumulation of struvite was observed by the authors in the exit of stabilization lagoons in a cassava processing industry.

The cultivation of microalgae in digested CPW is the most promising pathway for integration into biorefineries, using both the liquid residue and the CO_2 generated in biodigesters and/or in combustion. For light and carbon limited cultures, productivities are in the range of 15-30g.m^{-2}.day^{-1}. For high rate ponds with 100L/m^2, the optical path is similar to that used in laboratory and concentrations of 2-4g/L could be expected, with hydraulic retention times of 15 days. That means that an area of 10m^2 can treat the residue from more than 1 hectare of processed cassava:

10 m^2 x 100L/m^2 x 365days/15days = 24330L of CPW processed.

That requires only 0,1% of the planted area. This requirement can be even reduced if the culture system uses tanks in series and high inoculum concentrations or closed, high density bioreactor systems, with additional supply of CO_2 from biogas or flue gases. With microalgal production integrated to cassava processing, the bulk of

5.2 Microalgae cultivated in digested CPW

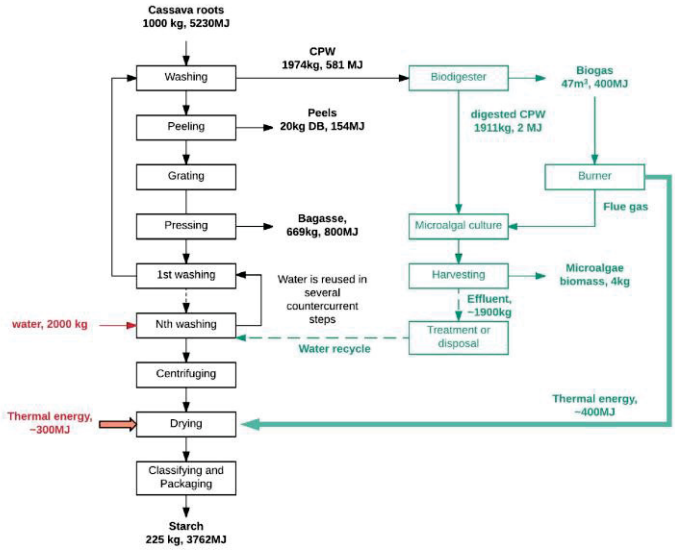
Cassava can be anaerobically digested to produce methane (biogas) or biohydrogen. In both cases, the fuel produced can be used in the industry, reducing the operational costs of the plant. For production of biogas, CPW is simply directed to a biodigester to be converted into methane; for production of biohydrogen, the biodigester must not have methanogens, and this is achieved by using a controlled inoculum population. Biogas yields were reported as about 0.3 to 0.6 L of biogas per gram of COD removed. Fleck et al. (2017) and Kuczman et al. (2016) obtained 90.4 to 99% reduction of COD in their CPW biodigester systems, with energy production ranging from 15.8 to 97.4 kWh/ton of CPW, the broad range being due to the different COD of the residues used.

There is a trend in researching hydrogen production in CPW, but the energetic balance shows that for in situ burning, methane is a more suitable fuel:

$C_6H_{12}O_6 \rightarrow 3CH_4 + 3CO_2$, ΔH_R = -127kJ/mol glu; the energy conservation is 95.5%

While hydrogen production is favorable only if followed by a methanogenic phase:

$C_6H_{12}O_6 + H_2O \rightarrow 4H_2 + 2CH_3COOH + 2\ CO_2$, ΔH_R = +96kJ/mol glu; the energy conservation is 41% to hydrogen, and the reaction is not thermodynamically favorable – that is, practical yields are lower than the theoretical stoichiometric maximum of 4mols H_2/mol glu. With another sequential reactor, the organic acids in the liquid stream may be converted (stoichiometrically) into 2 extra methane mols/mol glu, and the overall energy conservation rises. The literature describes yields from 1.34 to 2.41gH_2/mol glucose (Cappelletti et al., 2011), with higher COD concentrations corresponding to lower H_2 yields.



5.1 Microalgae production in pretreated CPW

CPW may have a large amount of suspended solids, and that reduces the light penetration in microalgal cultures. Therefore, a preliminary step of flocculation and sedimentation can be beneficial, and the resulting solid mass can be mixed with other residues such as peel for composting. Flocculation is often done in order to reduce the COD fed to the existent treatment systems (Oliveira et al., 2001). Although the reduction in dissolved organic carbon is minimal, the process greatly reduces turbidity, by 95%, and if followed by a microfiltration step, can reduce COD by 47% (dos Santos et al., 2017).

5. Integrating Microalgal production to modern cassava biorefineries

There are several alternatives for integrating microalgal cultures in cassava processing. The most promising are the cultivation in pretreated CPW, and the cultivation in anaerobically digested CPW. Figure 3 shows the main steps in starch production from cassava and the fate of the chemical energy in the roots, calculated based on process descriptions and yields for cassava processing from (Pandey et al., 2000)(De Carvalho et al., 2011)(Hansupalak et al., 2016), residue composition data (bagasse, peels) from (Woiciechowski et al., 2002)(F. Apata and O. Babalola, 2012)(Polachini et al., 2016), cassava nutritional composition and energy content from (Charrondière et al., 2017), and biogas yields and composition averaged from Fleck et al. (2017) and Kuczman et al. (2016)

Figure 3 – Material balance and energy content in starch production from cassava (in black), with integration of a biodigestion and microalgal biomass unit (in green). The inputs in red are note necessary in an integrated process.

toxicity was evaluated simulating the cyanogen environment with the addition of KCN in the autotrophic culture medium. The microalgae *Monoraphidium* sp. and *Scenedesmus* sp. showed the capacity to grow in medium containing up to 200ppm of KCN, whereas the microalgae *Chlorella* sp. and *Golenkinia* sp. withstood maximum concentrations of 40 ppm.

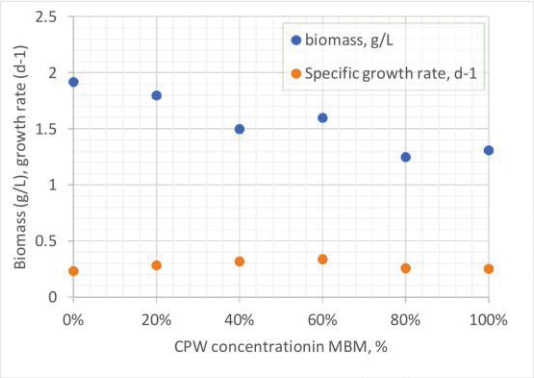
To evaluate the survival of the microalgae when cultivated in the liquid residue of the cassava processing, non-sterile CPW, sterile CPW and pre-treated CPW (anaerobic digestion) were used as raw material in different concentrations. Raw CPW was not suitable for cultivation in high concentrations, but was adequate when diluted (10%). Sterilized CPW, at the other side, was adequate up to 30% of concentration, while pure digested CPW supported the growth of *Monoraphidium* sp., *Golenkinia* sp., and *Scenedesmus* sp., and had to be diluted to 40% to support the growth of *Chlorella* sp. In these conditions, the cell counts reached 2, 4 and 7.10^5 cells/mL for the first three microalgae, and 1.10^5 for the latter.

Although microalgae production can be done directly in CPW, the amount of biomass produced is comparable to that obtained in autotrophic cultures. Because there is an important reduction of the BOD, it is clear that the organic carbon in CPW is being oxidized to CO_2 by part of the microalgal culture, possibly the bacterial population. This is adequate for wastewater treatment. However, if the organic carbon is not used by microalgae, why waste it in oxidation? Can the energy content in the CPW be used before microalgal cultivation?

Incremental adaptation was used by Walter (2011) for *S. platensis* and *C minutissima*. One of the most common artificial culture media for *Spirulina sp.* is that described by Zarrouk (1996), which has a very high concentration of some salts and is therefore frequently diluted to 50% or 20%, yet sustaining fair growth. This and the higher concentrations of phosphate, iron and organic nitrate in CPW may explain why *S. platensis* grew better in media added of CPW; all cultures were started on 20% Zarrouk medium, but 5% of the medium was replaced every 4 days with concentrated CPW (or synthetic medium). The microalga was capable of sustained growth in mixotrophic culture, with a biomass production of at least 2.5g/L, a specific growth rate of 0.41 day^{-1} and a productivity of 0.02 $g.L^{-1}.day^{-1}$ at the end of the culture (when wastewater concentration and cell density were higher). The same strategy was used by the author with daily medium substitution with filtered CPW, leading to 4.3g/L of biomass. Growth of *Spirulina sp.* in digested sago starch wastewaters was already observed by Phang et al. (2000) with continuous cultures giving a productivity of 0.096$g.l^{-1}.day^{-1}$. Muliterno et al. (2005) obtained biomass concentrations of up to 5.4$g.L^{-1}$ adding glucose to 50% Zarrouk medium, a controlled condition that shows the potential performance of mixotrophic cultures.

Isolation was found to be an adequate strategy for cyanide-resistant microalgal cultures by Cartas (2017), at DEBB-UFPR. Four microalgae were isolated from secondary effluents from a cassava processing industry, tentatively classified as strains of *Chlorella* sp., *Scenedesmus* sp. *Monoraphidium* sp. and *Golenkinia* sp. The amylolytic activity and cyanide toxicity were determined for each of the isolated microalgae strains. The presence of amylase was identified in *Monoraphidium* sp., *Golenkinia* sp. and *Scenedesmus* sp. when inoculated in 0.2% agar-starch solid medium. The cyanide

Figure 2 – *Chlorella minutissima* growth in different concentrations of CPW diluted with MBM medium



Shock adaptation was used by Borghetti (2009) and consisted of two weeks cultivation of *C. minutissima* in pure MBM medium (modified Bristol medium, NIES, 2017), followed by reinoculation into 20% CPW, another two weeks of cultivation, reinoculation in 40% CPW, and so on. With that strategy, (Figure 2) the maximum growth rate increased from 0.23d^{-1} with pure MBM to 0.34 d^{-1} with 60%CPW. However, the final biomass concentration decreased with increasing CPW content, possibly due to the toxicity of cyanide on the culture, which must be metabolized by the microorganisms; or due to an extreme concentration of a nutrient such as calcium; or due to the shadowing effect of suspended solids. This last possibility is reinforced by the results obtained in the adaptation of *S. platensis* and *C.minutissima* to CPW with and without filtration, done by Walter (2011). The author obtained 40% more *Spirulina platensis* and 45% more *Chlorella minutissima* biomass in filtered CPW, compared with cultures in the raw residue.

microalgae are regarded as GRAS (generally recognized as safe) and could be further used in animal nutrition studies. Both strains grow quickly, with specific growth rates in autotrophic cultivation on the order of $0.67d^{-1}$ for *Spirulina* sp. and up to $2.6d^{-1}$ for *Chlorella sp*. Autotrophic growth gives lower maximum growth rates – in the case of *Spirulina sp.* a third of the photosynthetic cultivation (Lee, 2004), therefore growth in CPW could show even higher growth rates.

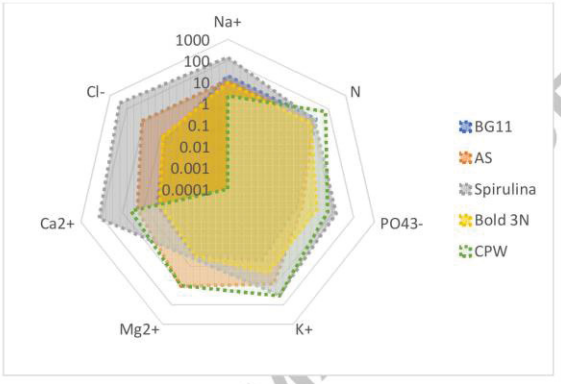
Bacterial contamination in unialgal starter cultures could develop with a complex dynamics: some microalgae can produce amylase inhibitors (Cannell et al., 1987) and therefore have a competitive edge over starch-degrading microorganisms; at the other side, the presence of nitrogen-fixing bacteria may be important when toxic amounts of ammonia are present, converting reduced forms of nitrogen into NO_3^-. Bacterial presence can also be beneficial for biodegrading the cyanide present in the effluents, as shown by Kandasamy et al. (2014).

Therefore, to test the growth of microalgae, the first step is to prepare several dilutions of the residue in different conditions: raw, filtered, and heat sterilized, and evaluate the growth of the microalga. Both *Spirulina platensis* and *Chlorella minutissima* grew in CPW diluted to 10% with water.

The second step in culture development is the adaptation of the microalgal culture, to select the most adequate dilution of CPW. This is necessary because some of the solutes may inhibit microalgal growth – especially ammonia and cyanide, for cultures poorly adapted. This adaptation can be done by incremental addition of CPW to a culture, or direct inoculation on CPW with the adequate concentration. The latter is easy to perform, but the shock can reduce cell viability.

high nutrient concentration. That is precisely what CPW has. Figure 1 shows the main components of CPW and those of popular microalgal culture media.

Figure 1 – Main nutrients in CPW and popular culture media. Values are in mg/L. BG11, AS, Spirulina and Bold 3N recipes are from UTEX (2017).



As can be seen in Figure 1, the concentrations of the main nutrients in culture media are variable, and comparable to those in CPW– except for chloride, which is a counterion in several media and not exactly a nutritional component. The media BG11 and AS have a stoichiometric excess of nitrogen, while Spirulina, Bold and CPW have an excess of phosphate, considering the ideal molar ratio near 16N:1P. In CPW, nitrogen is a mixture of organic and inorganic forms.

4.3 Microalgal cultures at the DEBB – UFPR, Brazil

In the beginning of our work with microalgae, several species were considered for growth in CPW, but two were selected for initial tests in larger cultures, indoors: *Spirulina platensis* LEB 52 and *Chlorella minutissima* LEB 108 (Borghetti, 2009). These

to convert CO_2 into organic carbon through photosynthesis. When inoculated in CPW, microalgae have simple sugars, mainly sucrose, glucose and fructose to use as carbon sources, and if the culture is illuminated, they will also grow photosynthetically.

4.1 Microalgae and the myth of axenic cultures

Most of the microalgae cultures are unialgal, but not axenic (Kraft and Fox, 2014), especially in the case of mass cultures (Borowitzka and Moheimani, 2013). Therefore, the growth of microalgae in any wastewater with organic carbon will lead to mixed cultures, and that is often beneficial in terms of wastewater treatment (Sen et al., 2013)(Fuentes et al., 2016). However, microalgae can outcompete bacteria in aerobic conditions amounting from typically 95 to 98% of the total microbial biomass (Passarge et al., 2006)(Cho et al., 2017), which means higher counts of bacteria but low bacterial biomass.

4.2 Microalgae cultivation in CPW

Microalgae occur naturally in stabilization ponds (Abeliovich, 2004), the final step before treated effluent release to water bodies. These microalgae actually contribute to provide oxygen as part of the oxidation process that takes place in these lagoons. There has been some euphory in the 80s regarding mass production of microalgae in wastewaters, but the problem pointed by Abeliovich (2004) is that efficient treatment systems require high efficiency in removal of nutrients – and therefore low concentrations and low productivities, while efficient biomass production demands

biosurfactants (Nitschke and Pastore, 2006), PHB (Sangyoka et al., 2012), aroma compounds (de Oliveira et al., 2013)(Damasceno et al., 2003), and microalgae (our group, see section 4). However, more recent research focus on anaerobic digestion of CPW (Khongsumran et al., 2014)(Cartas, 2017).

3.3 What is available, raw or digested CPW?

Anaerobic and facultative CPW lagoons in traditional treatments release large amounts of methane. Since ca. 11% of the energy content in cassava is lost to CPW, it is only logical that biogas be produced using CPW. Modern starch industries use this gas to feed the burners in flash dryers. It is estimated that biogas produced from CPW can be enough to supply all the energy a cassava processing industry needs, although that demands high capital costs and a few years for payback (Van Tran et al., 2017). In the past years, most of the cassava starch factories in Thailand, for example, switched from fossil fuels to biogas (Hansupalak et al., 2016).

Therefore, both raw and digested CPW are possible substrates for microalgae production. While the first is still available in several industries, the second still carries several nutrients and is more likely to be available in the future.

4. Microalgae production in CPW

Microalgae are capable of both autotrophic and heterotrophic growth. When cultivated in the presence of organic carbon, they may uptake these carbon sources as aerobic microorganisms do; in the presence of inorganic carbon, microalgae need light

popular in Northern Brazil. It is detoxified by action of linamarase, which frees cyanide from the glucoside, and subsequent boiling, since HCN is volatile (Cohen et al., 2007)(FAO, 2013).

Cyanide disrupts the respiratory transport chain. It is fatal at low doses (1-2mg/kg) for mammals (O'Neil, 2013). Plants have higher resistance to cyanide, but the compound also affects the Calvin-Benson cycle (the dark reactions responsible for carbon fixation) (Hill et al., 2014) because inhibits the carbon supply by carbonic anhydrase, as observed e.g. in *Chlorella pyrenoidosa* (Jahnke, 1981), and presumably inhibits the electron transport chain in the photosystem II (Packham et al., 1982). In fact, toxicity is observed for microalgae, and selection of species is necessary prior to cultivation.

3.2 CPW treatment and uses

Direct use of CPW as a fertilizer is limited and may lead to phytotoxic effects. The same goes for animal feed – CPW can be used to a limited extent depending on the cassava variety that is being processed. These practices are also limited by transportation costs.

The typical CPW processing consists of sieving, a primary sedimentation for separation of sand and other particulates, then an anaerobic lagoon, and a facultative lagoon. The residual water is still rich in nitrogen and phosphate, and microalgal growth can usually be observed in the final steps of the treatment.

Because CPW has a fair amount of soluble sugars, several authors have been trying to use it as a substrate for the heterotrophic production of bioproducts such as

As can be seen in Table 2, the polluting or eutrophication potential of CPW is enormous. This is a very rich residue that, as a culture medium for microalgae, is rather nutritive, although presenting an stoichiometric excess of nitrogen – the proportion of nutrients generally recognized as adequate is around 100:16:1 of C, N and P (Tett et al., 1985).

Because of the presence of cyanide, which has a broad-spectrum toxicity, and because of the high chemical oxygen demand, the residue must be processed in the industry. Several *in situ* valorization alternatives have been investigated over the years, such as feed supplement, pesticides and fertilizers (with some success) and for citric acid (Leonel and Cereda, 1995) ethanol (Camili, 2007)(Suman et al., 2011), aroma compounds and biosurfactants (Damasceno et al., 2003)(Pastore et al, 2003), enzymes (Bicas et al., 2010) and volatile fatty acids in an ethanol biorefinery (Xie et al., 2014).

3.1 CPW Toxicity

All parts of the cassava plant have cyanide in the form of cyanogenic glucosides, mainly linamarin (95%). These glucosides are in cell vacuoles and liberated when the tissue is destroyed (Pfister, 2008) in flour or starch production. Because starch is washed, its cyanide content is very low, typically below 10 ppm and often undetectable. In flours, the cyanide content is higher and may typically range from 10 to 20 ppm, but can reach 100ppm (Djazuli and Bradbury, 1999)(Cohen et al., 2007). Conversely, starch industries have more of the cyanide removed to the CPW.

Oddly enough, there is a traditional sauce made with fermented CPW from flour production – the most concentrated kind of CPW. This sauce, called *tucupi*, is very

Table 2 – Composition of cassava processing wastewater from starch and flour industry

Reference → Components	*(Hien et al., 1999)*	*(Damasceno et al., 2003)*	*(da Silva et al., 2005)*	*(Nitschke and Pastore, 2006)*	*(Borghetti, 2009)*	*(Ouephanit et al., 2011)*	*(Sangyoka et al., 2012)*	*(de Oliveira et al., 2013)*	*(Khongsumran et al., 2014)*	*(Xie et al., 2014)*	*(Cartas, 2017)*	*(dos Santos et al., 2017)*	*(dos Santos et al., 2017)*	Average
Total carbohydrates (g/L)		58.2		35.3						5.2				32.9
Protein (g/L)										1.53				1.5
Reducing sugars (g/L)		38		12.8		6.07		0.57						14.4
Non-reducing sugars (g/L)				22.2		14.98								18.6
Total nitrogen (g/L)		1.6	0.032	2.5	0.154		1.41	0.16	2.67	0.24		0.098	0.07	0.9
Phosphorus (mg/L)		80	17.8	225.9	19.26		3300		80	120				549.0
Potassium (mg/L)		895	333.6	2665.1	1463									1339.2
Calcium (mg/L)		184	31.37	272.5										162.6
Magnesium (mg/L)		173	36.87	519										243.0
Sulfur (mg/L)	9	38		104										50.3
Iron (mg/L)		8	6.9	7.8										7.6
Zinc (mg/L)			0.59	7.3	3.6									3.8
Manganese (mg/L)			0.62	1.8										1.2
Copper (mg/L)		0.8	0.05	0.6										0.5
Sodium (mg/L)			51.7											51.7
pH	5.46	5.5	4.8	5.9	4.4	6.21	4.6	5.1	4.34	4	6	6.8	6.7	5.4
Soluble COD (g O2/L)							12.5		9.41	19.4				13.8
COD (g O2/L)	15.5	60	14.7	55.82	6.18	20.43	19.85	16.09	10.56		15.82	6.8	5.92	20.6
TDS						13.52								13.5
TSS	5.9	62			40.5	1.91		3.54			2.59	6.5	0.995	15.5
VSS								3.14			2.56			2.9
NH4, mg/L	174							14.4	2	8	8.7			41.4
NO3, mg/L					11.07				46.67		15.07			24.3
Cyanide, mg/L	5.6		12		62							1.9	0.67	16.4

Cassava processing wastewater (CPW) is the most important residue in terms of volume, wasted chemical energy and polluting potential, and evaluating its properties in a given process is the first step for defining uses for the residue.

3. CPW – composition and uses

Cassava processing waste has a variable composition depending on the crop – whose starch and cyanide content varies – and in the processing. Taking out starch and fibers, the root still has 60% of water and other compounds that will be mixed with variable amounts of process water and constitute CPW (Table 2). Therefore, the COD (Chemical Oxygen Demand) of the residue varies from 6 to 56g/L, but the C:N:P ratio is more predictable, at about 330:30:1 in a molar basis.

The residues from cassava processing for starch production are basically a small amount of peels, a larger amount of bagasse (fibers from the roots) and large amounts of wastewater. Most of the energy contained in the roots (ca. 120kcal/100g) is recovered as starch, amounting to about 70% of the initial energy content. The bagasse contains 15% of the edible calories of the root, with an energy content of 29kcal/100g, and the wastewater carries 11% of the edible calories (energy content 7kcal/100g), together with residual fibers and lignin. This wasted biomass and energy represents an opportunity for bioprocessing, because most of this chemical energy is easily convertible by microorganisms. Using these residues also prevents them of contributing to pollution, represents a possible income source, and can potentially reduce the energy and nutrient usage if properly recycled.

Several uses have been proposed for cassava residues. In small scale production, the peels can be composted, the bagasse can be composted or used for animal feed, and the leaves are left in the field, used for animal feed, or for human food. Liquid residues are produced in relatively small amounts and can be degraded in simple lagoons or biodigesters.

Larger operations generate large concentrated amounts of solids, and the agroindustrial residues must be better used. Several products of solid substrate fermentation using cassava bagasse have been proposed over the years (Carvalho et al., 2007)(Trakarnpaiboon et al., 2017)(Sugumaran et al., 2014)(Pengthamkeerati et al., 2012)(Prado et al., 2005)(Pandey et al., 2000).